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TÍTULO DEL TRABAJO:

PARTICIPACIÓN DE P53 EN LA APOPTOSIS MEDIADA POR CD95 Y MG132 EN QUERATINOCITOS HUMANOS INMORTALIZADOS CON E6 DEL VPH16

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1. ABREVIATURAS

AIF	Apoptosis-inducing factor
APAF-1	Apoptosis protease-activating factor-1
APO-1	Apoptosis antigen-1
CaCU	Cáncer cervicouterino
DBD	DNA binding domain
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DMSO	Dimetilsulfóxido
DR4	Death receptor 4
FADD	FAS-associated death domain
LC3	Light chains-3
LCR	Long control región
ORF	Open reading frame
PFT-α	Pifithrin alpha
qPCR	Quantitative polymerase chain reaction
TAD	Transactivation Domain
TNF	Tumor Necrosis Factor
TNF-R1	Tumor Necrosis Factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
VPH-AR	Virus del Papiloma Humano de alto riesgo

2. ABSTRACT

The E6 oncoprotein can interfere with the ability of HPV infected cells to undergo programmed cell death through the proteolytic degradation of proapoptotic proteins such as p53, FADD or procaspase-8, employing the proteasome pathway. Therefore, inactivation of the proteasome through MG132 should restore the activity of several proapoptotic proteins. We investigated whether in HPV16 E6-expressing keratinocytes (KE6 cells), the restoration of p53 levels mediated by MG132 and/or activation of the CD95 pathway is responsible for the induction of apoptosis. We found that KE6 cells underwent apoptosis after incubation for 24 h with either MG132 alone or MG132 plus APO-1. Both treatments activated the extrinsic and intrinsic apoptosis pathways and also affected the mitochondrial membrane potential. Similar to apoptosis, autophagy was also activated, principally by MG132 plus APO-1. Inhibition of E6-mediated p53 proteasomal degradation by MG132 resulted in elevated p53 and phospho-p53 Ser46 and Ser20 protein levels. In addition, induction of its transcriptional target genes such as p21, Bax and TP53INP were observed 3 and 6 hours after treatment. Also, LC3 mRNA was induced after 3 and 6 hours. Finally, using pifithrin alpha we observed a decrease in apoptosis induced by MG132, and by MG132 plus APO-1, suggesting that restoration of APO-1 sensitivity occurs in part through an increase in both the levels and the activity of p53. The use of small molecules to inhibit the proteasome pathway might permit the activation of autophagy and apoptosis pathways, providing new opportunities for cervical cancer treatment.

3. RESUMEN

La oncoproteína E6 inhibe la apoptosis a través de la degradación de proteínas proapóptoticas, tales como: p53, FADD o procaspasa-8, utilizando la vía del proteosoma. La inactivación del proteosoma a través de inhibidores de este complejo (como MG132), debería de restaurar la actividad de proteínas proapóptoticas. En este trabajo, se investigó sí en queratinocitos humanos que expresan a la oncoproteína E6 del VPH16 (QE6-16), el restablecimiento de los niveles de p53 mediados por MG132 y/o activación de la vía de CD95 es responsable de la inducción de la apoptosis. Los resultados muestran que QE6-16 cultivados en presencia de MG132 o APO-1 más MG132 son inducidos a apoptosis después de 24 h. De forma similar, la autofagía también fue activada, principalmente por APO-1 más MG132. La degradación de p53 a través de E6 fue inhibida por MG132, resultando en niveles incrementados de p53 y su fosforilación en Ser20 y Ser46 detectando la proteína p53 en el núcleo, con los tratamientos MG132 y APO-1 más MG132. Además, se incrementó la expresión del RNA mensajero de p21, Bax y TP53INP a las 3 y 6 h de tratamiento. Utilizando pifitrina alfa (inhibidor de la actividad transcripcional de p53), se observó una disminución en la apoptosis inducida por MG132 y APO-1 más MG132, sugiriendo que la restauración de la sensibilidad a APO-1 es debida principalmente por los niveles incrementados y la actividad de p53. El uso de MG132 podría inducir la activación de autofagía y apoptosis generando nuevas opciones de tratamiento del CaCU.

4. INTRODUCCIÓN

4.1. Cáncer cervicouterino.

El cáncer cervicouterino (CaCU) constituye uno de los principales problemas de salud pública en mujeres. Actualmente, el CaCU es considerado la cuarta causa de cáncer en mujeres a nivel global y en México es considerado el segundo tipo de cáncer en mujeres (Ferlay y col., 2013; Bruni y col., 2016). A nivel mundial se registran 527,624 casos nuevos y 265,672 muertes por CaCU al año; en México se registran 13,900 casos nuevos y 4,769 muertes por esta causa al año (Ferlay y col., 2013; Bruni y col., 2016). El Virus del Papiloma Humano (VPH) ha sido identificado como el agente etiológico del CaCU (Schlecht y col., 2001; Londesborough y col., 1996). Sin embargo, la infección por el VPH de alto riesgo (VPH-AR) no es suficiente para el desarrollo del CaCU, por lo que se requieren diversos factores genéticos, ambientales y biológicos (Martínez-Nava y col., 2016; Au, 2004; Lee y col., 2004; Roura y col., 2016).

4.2. Biología molecular del Virus del Papiloma Humano.

Los VPHs son virus pequeños que carecen de envoltura, su genoma se encuentra formado por un ADN circular de doble cadena de aproximadamente 8000 pares de bases (pb) (Doorbar y col., 2015). Actualmente se han reportado 170 tipos de VPHs; de los cuales un tercio de ellos infecta específicamente células del tracto genital (De Villiers, 2013). De acuerdo a su potencial oncogénico los VPHs se han clasificado en dos grupos: 1) los VPHs de alto riesgo (VPH-AR: 16, 18, 31 y 58, entre otros); 2) y los de bajo riesgo (VPH-BR: 6 y 11, entre otros) (Muñoz y col., 2003). Los VPH-AR se encuentran asociados con lesiones que pueden progresar a neoplasia intraepitelial de alto grado y finalmente a CaCU (Joura y col., 2014).

Todos los genomas de los VPHs presentan tres regiones bien definidas: 1) una región larga de control (URR o LCR, por sus siglas en inglés), a través de la cual se regula la replicación y transcripción viral. 2) Una región temprana que contiene los marcos de lectura de expresión temprana E1, E2, E4, E5, E6 y E7. 3) La región de expresión tardía la cual codifica para los genes de expresión tardía L1 y L2, que conforman la cápside viral (Figura 1) (Doorbar y col., 2012).

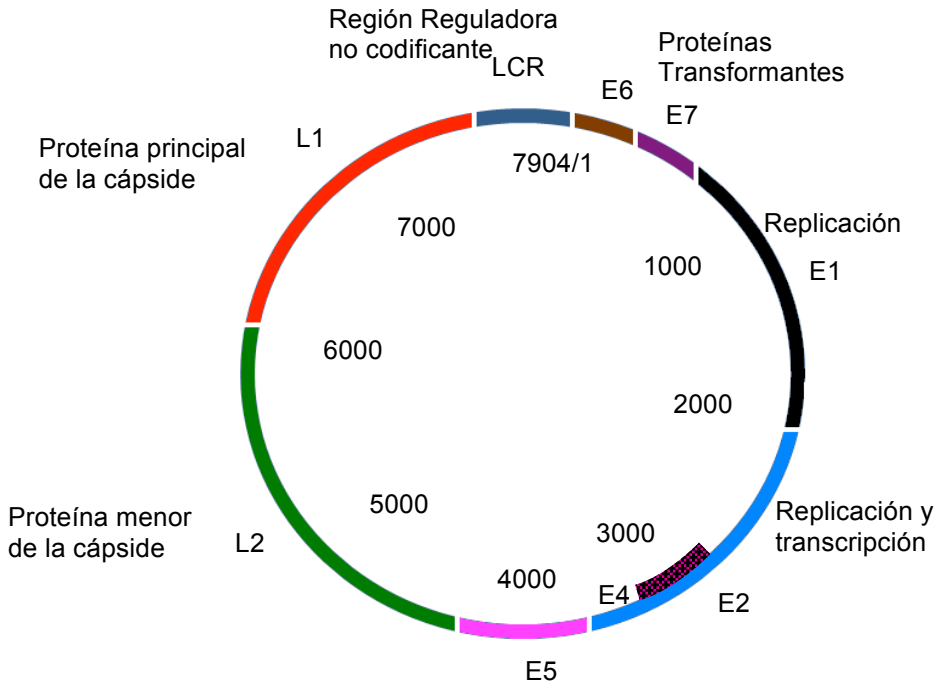


Figura 1.- Imagen representativa de la organización del genoma del VPH-16. La estructura genómica comprende la LCR y ocho genes que son necesarios para diferentes estadios del ciclo viral. La LCR contiene sitios de unión para factores de transcripción celulares (ej., SP1, AP1, Oct1), así como para las proteínas virales E1 y E2; las cuales controlan la replicación y expresión viral. Se indica en números la región que comprende cada gen en el genoma viral.

A continuación se describirán de manera breve la función de las proteínas virales no estructurales.

La proteína viral E1 participa en el reconocimiento del origen de replicación del genoma del VPH. Esta proteína (en colaboración con la proteína E2), se une a elementos específicos del ADN viral en el origen de replicación, formando helicasas hexaméricas. El complejo E1-E2 relaja las hebras del ADN en el sitio de origen de la replicación viral favoreciendo la síntesis del ADN (Lehoux y col., 2014; Bergvall y col., 2013). Además, la proteína E1 interacciona con diversas proteínas celulares que participan en replicación del genoma celular, reclutándolas al origen viral, como es el caso de la ADN polimerasa alfa y la proteína de replicación A (RPA, por sus siglas en inglés) (Bergvall y col., 2013).

La proteína E2 es requerida para la replicación viral y regulación de la expresión de los genes virales (McBride y col., 2013; Graham y col., 2016). La función clásica de las proteínas E2 es

la regulación transcripcional de E6 y E7 a través de su unión a los elementos de respuesta de E2 en la LCR. (Bernard y col., 1989). Adicionalmente, se ha encontrado que las proteínas E2 de los VPH-AR presentan propiedades relacionadas con transformación celular, tales como inducción de mitosis anormales que deberían guiar a eventos de aneuploidía y/o ruptura de hebras de ADN (Bellanger y col., 2005; Kadaja y col., 2007; Kadaja y col., 2009; Tan y col., 2015). Además, E2 se une al genoma viral episomal y a la cromatina celular, eventos que podrían favorecer el acercamiento entre ambas moléculas; y a través de la fragmentación de las hebras del ADN genómico inducido por E2 durante la mitosis facilitar la integración del ADN viral en el genoma celular (Bellanger y col., 2011). Sin embargo, la disrupción del marco de lectura (ORF, por sus siglas en inglés) de E2, frecuentemente ocurre durante la integración del genoma del VPH al genoma celular sugiriendo que la participación de E2 no es requerida para la carcinogénesis del cuello uterino (Kahla y col., 2014).

Diversas publicaciones han sugerido que las proteínas virales E4 y E5 son sintetizadas en la fase tardía del ciclo viral del VPH, siendo la proteína E4 la más expresada de todas las proteínas virales en los epitelios infectados (Stoler y col., 1992; Supchokpul y col., 2011). El ORF de E4 es generado de un transcrito que presenta la región que codifica para los primeros 5 aminoácidos de la proteína viral E1, generando la proteína de fusión E1ˆE4 durante la fase tardía del ciclo viral (Doorbar, 2013). La proteína E1^E4-AR se asocia con redes de queratina en las células (Wang y col., 2004). A través de ensayos de transfección se ha observado que cuando la proteína E1^E4 es sobreexpresada puede llegar a inducir el colapso celular (Doorbar y col., 1991). Este evento podría ser favorecido por la inducción de apoptosis de E1^E4 al asociarse con la mitocondria (Raj y col., 2004). Sin embargo, en infecciones por los tipos virales de AR en epitelios, solamente una cantidad limitada de colapsos celulares se han observado. También, la proteína E4 interacciona con la RNA helicasa E4-DBD (E4-DEAD box protein), la cual es un miembro de una familia de helicasas involucradas en el procesamiento del ARN, transporte y traducción, sugiriendo que E4 podría jugar un papel en la regulación de la expresión génica (Longworth y Laimins, 2004).

E5 es una proteína hidrofóbica unida a la membrana que se asocia con el aparato de Golgi, retículo endoplásmico, y membrana perinuclear (DiMaio y Petti, 2013). En estadios tempranos de la infección por el VPH-16, el ARN mensajero de E5 es uno de los transcritos virales más abundantes (Stoler y col., 1992). Varios mecanismos podrían ser activados para favorecer el

desarrollo del CaCU por parte de E5. Por ejemplo, ha sido propuesto que E5-AR inhibe la apoptosis mediada por receptores de muerte (FAS, DR4 y DR5) en queratinocitos humanos, evento que podría estar ocurriendo en los estadios tempranos de la infección viral (Kabsch y Alonso, 2002). Además, E5-AR favorece la activación de la vía del receptor del factor de crecimiento epidermal (EGF-R) y vías de señalización relacionadas con inflamación (Crusius y col., 1998; DiMaio y Petti, 2013). Estos eventos podrían contribuir de forma inicial a que E5 favorezca la progresión del CaCU a través de modular las vías de señalización, aumentando el potencial oncogénico de E6 y E7.

Las oncoproteínas virales E6 y E7 participan en el proceso de transformación e inmortalización celular. Concretamente, muchas proteínas celulares interaccionan y son degradadas por la proteína viral E6, como es el caso de la proteína supresora de tumores p53, a través de un mecanismo dependiente de ubiquitina (Scheffner y col., 1990). La proteína E7 favorece la degradación de la proteína supresora de tumores pRb (Scheffner y col., 1992). La integración del genoma viral en el cromosoma celular resulta en la perdida de la expresión del represor viral E2, evento que induce alta expresión de las proteínas E6/E7 (Steger y Corbach, 1997; Kahla y col., 2014). Ha sido reportado que las oncoproteínas E5, y principalmente E6 y E7 son necesarias para el mantenimiento del fenotipo transformado (Maufort y col., 2010; Münger y col., 1989), aunque su sola expresión no es suficiente para transformar células humanas, alteraciones celulares también son requeridas.

4.3. La proteína E6 de VPH-AR.

Las proteínas virales E6-AR están integradas por aproximadamente 150 aminoácidos y presentan 2 dedos de Zinc unidos por un interdominio de 36 aminoácidos (Figura 2)(Wallace y Galloway, 2015). E6-AR juega un papel importante en la carcinogénesis del cuello uterino a través de transformación e inmortalización celular (activando a la telomerasa). Además, E6 altera procesos tales como apoptosis, respuesta inmune, inestabilidad cromosómica, diferenciación celular y proliferación. Estos eventos celulares son desarrollados a través de interacciones proteicas, con diversas proteínas (Bak, CBP/p300, c-Myc, E6TP1, hADA3, IRF3, MCM7, PTPH1 y TNF-R1) (Tungteakkhun, y col., 2008; Lagunas-Martínez y col., 2010). La unión a estas proteínas es realizada a través de un motivo conservado (LXXLL) en

la proteína E6, el cual ha sido relacionado con la unión a proteínas celulares que contienen dominios PDZ y E6AP, y algunas otras que no tiene un dominio de unión conocido con la proteína E6, como son p300/CBP y hADA3 (Ganti y col., 2015).

Uno de los principales mecanismos a través de los cuales se favorece la transformación e inmortalización celular es la inhibición de la muerte celular. La oncoproteína E6 es una de las proteínas virales que inhibe la apoptosis celular inhibiendo la activación de la vía intrínseca o extrínseca (Lagunas-Martínez y col., 2010).

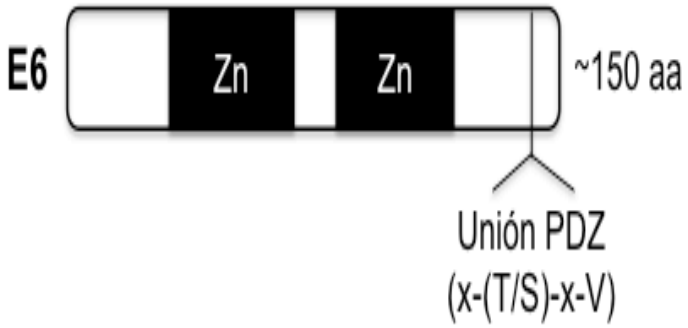


Figura 2. Estructura de la proteína viral E6. Se muestra la localización de los dedos de zinc (regiones de color negro) y la región de unión a las proteínas PDZ (Ganti y col., 2015).

4.4. Tipos de Muerte Celular.

a) Apoptosis (Muerte celular tipo I)

La apoptosis es un tipo de muerte celular programada (tipo I) en la cual de forma sistemática se activa una cascada de activación de proteasas que fragmentan una gran cantidad de proteínas celulares. La apoptosis se divide en vía intrínseca y extrínseca (Mariño y col., 2014; Green y col., 2014).

La vía extrínseca se activa a través de la unión de ligandos a los receptores de muerte. Tres de las vías más comunes son la vía de TNF, FAS (CD95) y TRAIL. Una de las vías más estudiada es la vía de FAS la cual se describirá a continuación (Green y Llambi, 2015).

Posterior a la unión de ligando a su receptor, el receptor se trimeriza favoreciendo la unión de la proteína adaptadora FADD al dominio de muerte (DD, por sus siglas en inglés) del receptor. Este evento favorece el reclutamiento de procaspasa-8. La interacción de estas proteínas forma el complejo de señalización inductor de muerte (DISC). La activación de caspasa-8 favorece la activación de caspasa-3 y de manera posterior la fragmentación de proteínas blancos de esta proteasa tales como actina, lamina A, PARP, entre otras (Kantari y Walzcak, 2011).

La vía intrínseca se activa a través de estímulos internos tales como estrés oxidativo, generación de radicales libres, daño al ADN, entre otros. Los diferentes estímulos que recibe la célula favorecen la permeabilización de la membrana mitocondrial favoreciendo la liberación de proteínas que sensibilizan a la célula a muerte celular, tales como citocromo c (Cyt-c), SMAC/DIABLO, AIF, ENDO G, entre otras (Brenner y Wak, 2009). Posterior a su liberación de la mitocondria, Cyt-c se une a la proteína Apaf-1, reclutando posteriormente a procaspasa-9. La formación de este complejo se denomina apoptosoma. Activada caspasa-9 favorece la activación de caspasa-3 y finalmente la muerte de la célula (Figura 3) (Würstle y col., 2012). La apoptosis puede comenzar con la autofagia y la autofagia puede terminar a menudo con la apoptosis (Booth y col., 2014).

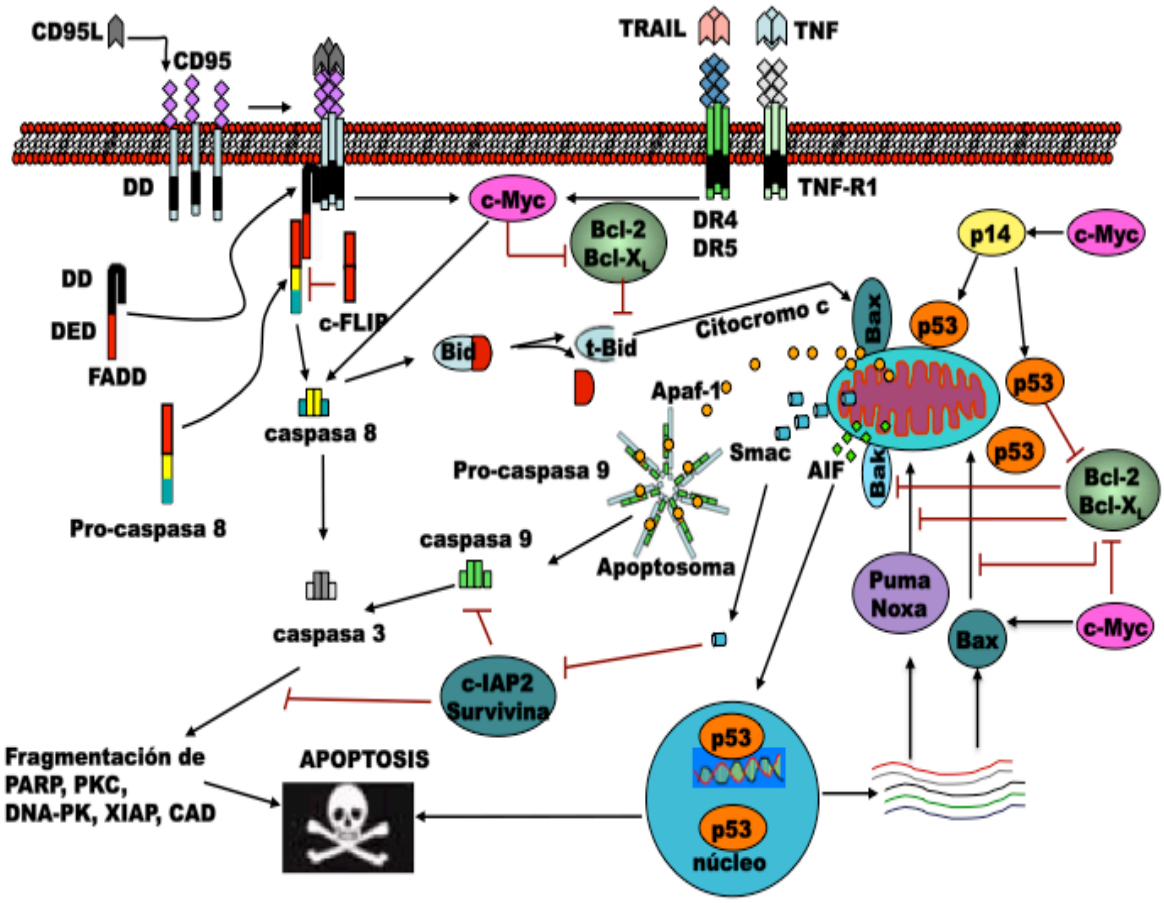


Figura 3. Mecanismos de regulación de la Apoptosis (Muerte celular tipo I). En la vía extrínseca, CD95L se une a su receptor CD95 induciendo su trimerización. CD95 recluta a FADD a través de interacciones con el dominio de muerte (DD, por sus siglas en inglés). Posteriormente, FADD recluta a procaspasa-8, formando el complejo denominado complejo de señalización inductor de muerte (DISC, por sus siglas en inglés). Activada caspasa-8, induce apoptosis a través de la activación de caspasa-3. En la vía intrínseca, la proteína Bax es translocada de diversas regiones del citoplasma a la membrana mitocondrial, favoreciendo la liberación de AIF, citocromo c y Smac al citoplasma. Citocromo c se une a Apaf-1 induciendo la activación de caspasa-9, y finalmente la activación de caspasa-3/7. p53 induce la apoptosis a través de la activación transcripcional de sus genes blanco proapoptóticos en el núcleo, o a través de la interacción directa con proteínas anti- y proapoptóticas en mitocondria. De igual forma, c-Myc induce apoptosis a través de: 1) activación de p53 mediado por p14 favoreciendo la activación de Bax; 2) a través de la activación de Bax de forma directa; además, 3) amplifica la señal de caspasa-8 mediada por activación de receptores de muerte. Rectángulos en color negro indican dominios de muerte; y rectángulos en color rojo, dominios efectores de muerte (Modificada de Lagunas-Martínez y col., 2010).

b) Autofagia (Muerte Celular Tipo II)

La autofagia (muerte celular tipo II) es un mecanismo activado de forma natural para reciclar los componentes celulares y mantener un balance de los nutrientes celulares y la función de los organelos (Pavel y Rubinsztein, 2016). La autofagia puede ser activada por diversas condiciones fisiológicas que comprenden muerte celular, eliminación de células tumorales, eliminación de patógenos y presentación de antígenos (Korolchuk y col., 2011). La autofagia puede ser también activada por radiación o compuestos citotóxicos. En determinadas condiciones, algunos organelos del citoplasma son enviados a vesículas de doble membrana denominadas autofagosomas, las cuales se fusionan con lisosomas para degradación de proteínas por hidrolasas lisosomales (Klionsky y Emr, 2000). La formación de autofagosomas es un proceso finamente regulado que requiere más de 30 proteínas, muchas de las cuales son conocidas como proteínas ATG. Entre estas proteínas un elemento clave es LC3. La cadena ligera 3 de la proteína 1 asociada a micro túbulos (LC3, por sus siglas en inglés) es localizada en los autofagosomas y autofagolisosomas. La proteína LC3 de humano presenta 3 isoformas (LC3A, LC3B y LC3C). LC3 es convertido a LC3-I después de que el dominio carboxilo terminal se escinde para exponer un residuo de glicina (Wesselborg y Stork, 2015). Este proceso convierte a LC3 en un mecanismo de conjugación similar al generado por ubiquitina. LC3 puede ser entonces conjugada a través de su glicina con el lípido fosfatidiletanolamina, en una reacción que requiere diversas proteínas ATG, las cuales tiene un mecanismo de acción parecido a las moléculas E1-E3 de la conjugación de la ubiquitina. La lipidación de LC3 (LC3-II) permite la expansión y cierre de las membranas del autofagosoma (Kabeya y col., 2000; Pavel y Rubinsztein, 2016). Es probable que el primer marcador molecular de autofagia sea la modificación postraduccional de LC3B a través de lipidación (Figura 4). Un elemento clave en la regulación de la autofagia es p53. Como se ha descrito previamente, cuando p53 se encuentra en el citoplasma induce apoptosis pero no autofagia; sin embargo, cuando p53 se encuentra en el núcleo induce apoptosis y autofagia (Figura 5) (Green y Kroemer, 2009).

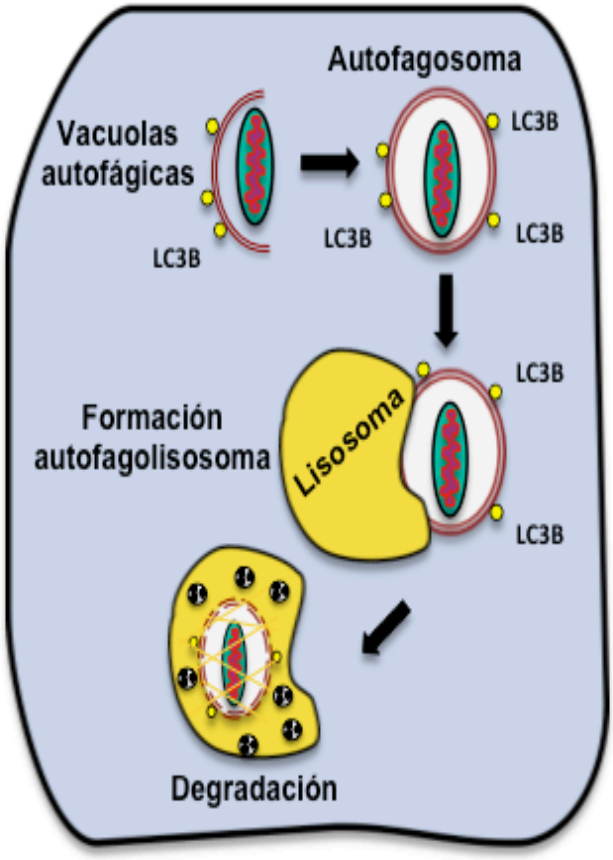


Figura 4.- Mecanismo de regulación de Autofagia (Muerte celular tipo II). Debido a diversos factores externos, la autofagia puede ser activada. Inicialmente se forman los autofagosomas, los cuales se unen con los lisosomas generando los autofagolisosomas. La presencia de LC3B lipidado en los autofagosomas es una modificación postraduccional clave en el inicio de autofagia.

4.5. Estructura y función de p53. Regulación de la muerte Celular.

El gen p53 (*TP53*) es considerado un Gen Supresor de Tumores debido a que regula el ciclo celular inhibiendo la proliferación descontrolado de células transformadas e inmortalizadas a través de la inducción de la muerte celular de estas. El gen p53 está integrado por 11 exones que codifican para una proteína de 393 aminoácidos (53 kDa) (Belyi y col., 2010). p53 es un factor de transcripción que en condiciones basales se encuentra en bajos niveles proteicos, difícilmente detectables debido a que su vida media es corta. Una proteína celular que regula la vida medía de p53 es la E3 ubiquitina ligasa HDM2 (Shen y col., 2013). En células infectadas por el VPH, la proteína viral E6-AR actúa como E3 ubiquitina ligasa regulando los niveles de diversas proteínas proapoptóticas (entre ellas p53)(Martinez-Zapien y col., 2016). Cuando las células se exponen a estrés celular, como daño al ADN, hipoxia y activación de proteínas oncogénicas, el nivel de la proteína p53 aumenta rápidamente debido a la regulación a nivel postraduccional que presenta (fosforilación y acetilación) (Lavin y Gueven, 2006). Posterior a estas modificaciones en p53, la célula podría ser guiada a diferenciación celular, reparación del ADN, senescencia, apoptosis (Liu y Xu, 2011; Meek, 2015). p53 actúa de dos formas en respuesta al daño al ADN. En la primera, detiene el ciclo celular en la fase G1 permitiendo que se repare el ADN antes de que se replique. En la segunda, induce muerte celular (apoptosis, senescencia o autofagia) cuando el daño genético ha sido severo e imposible de reparar (Kruiswijk y col., 2015). Las fosforilaciones de p53 están relacionadas con la estabilidad de la proteína (en Ser15 y Ser20) e inducción de apoptosis (Ser46) (Gu y Zhu, 2012; Meek, 2015). Posterior a la señal de muerte, p53 es localizado en la mitocondria, interaccionando e inhibiendo la actividad de la proteína antiapoptótica Bcl-2; o transloca al núcleo para inducir la transcripción de genes blanco que participan inhibiendo el ciclo celular (como p21) o que activan la apoptosis (Puma, Noxa, TP53INP1, Bax) (Figura 5) (Tang y col., 2015).

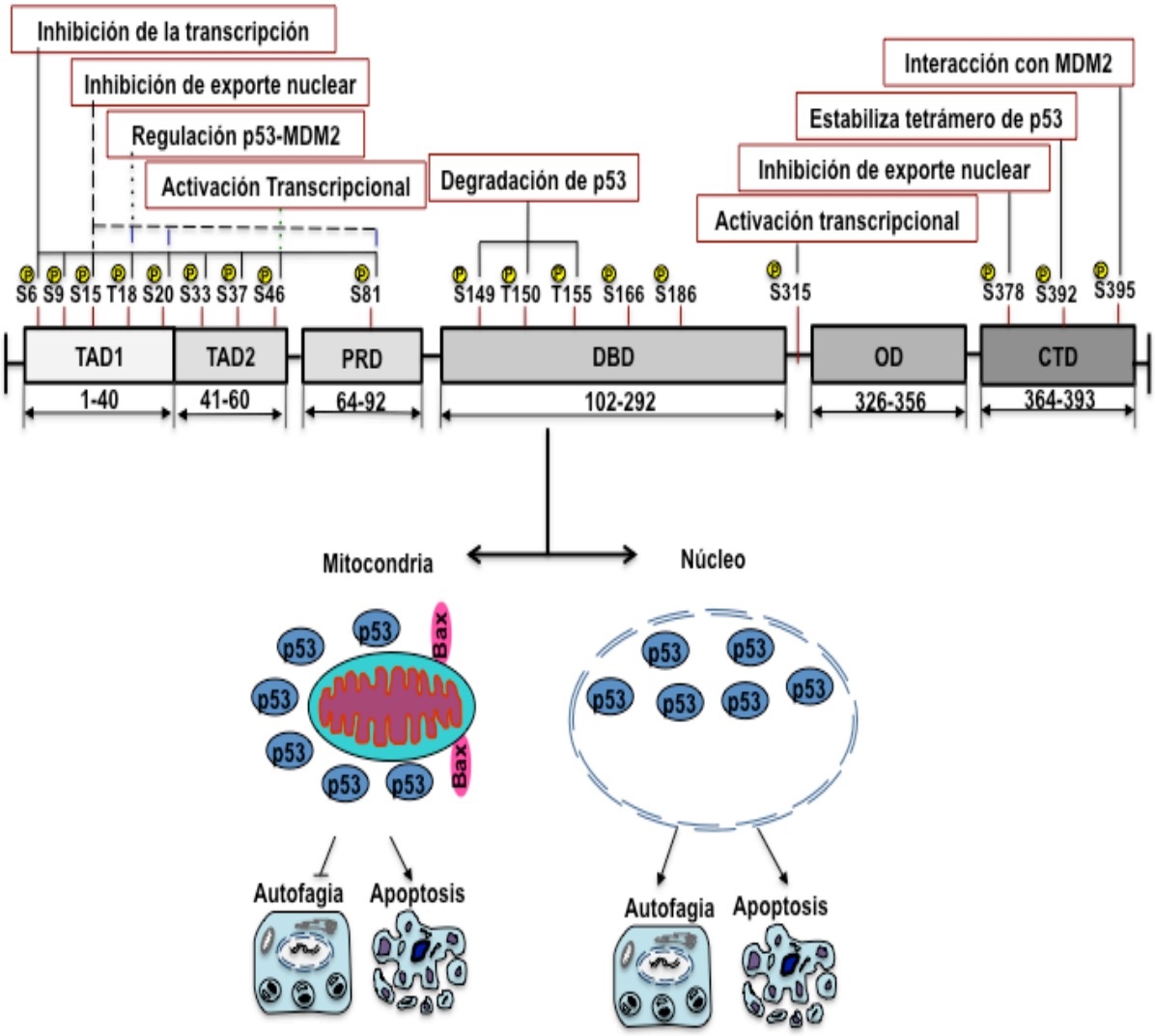


Figura 5. Estructura de la proteína p53 e inducción de la muerte celular. La proteína p53 está integrada por diversos dominios, como es el de transactivación (TAD), dominio rico en prolina (PRD), dominio de unión al DNA (DBD), dominio de oligomerización (OD), y el dominio carboxilo terminal. Los círculos en amarillo indican la posición de los aminoácidos fosforilados y las funciones asociadas a estas modificaciones postraduccionales. La localización celular de p53 regula el tipo de muerte celular que se activa. p53 induce a apoptosis y autofagia cuando se localiza en núcleo; sin embargo, si se localiza en mitocondria induce apoptosis pero no autofagia.

4.6. Estructura y función de c-Myc, regulación de Apoptosis.

El gen c-Myc está integrado por tres exones y dos intrones, el primer exón parece tener una función reguladora de la expresión del gen. La proteína se genera a partir del segundo y tercer exón (Wierstra y Alves, 2008) conformada por 439 aminoácidos (65 kDa) y una vida media de aproximadamente 20 min (Farrell y Sears, 2014). Los extremos amino y carboxilo terminal de c-Myc presentan varios dominios, los cuales son necesarios para las diferentes funciones celulares como unión al DNA, transactivación, oligomerización de la proteína, regulación del ciclo celular, proliferación, diferenciación celular y apoptosis (McMahon, 2014; Conacci-Sorrell, y col., 2014; Bretones y col., 2014). c-Myc posee un motivo de dimerización, denominado hélice-asa-hélice/cierre de leucina (HLH/LZ, por sus siglas en inglés) en el extremo carboxilo terminal (CTD), el cual consta de aproximadamente 90 aminoácidos (Conacci-Sorrell y col., 2014). Este dominio se requiere para la dimerización de c-Myc con otras proteínas celulares, como Max, la cual es requerida para formar un heterodímero y unirse al DNA activando mecanismos de regulación transcripcional (Cascón y Robledo, 2012; Conacci-Sorrell y col., 2014). El segundo dominio comprende el dominio de transactivación (TAD), en la región amino terminal (NTD) de c-Myc, la cual favorece la activación de genes blanco de c-Myc, (LDH-A, HIF, p73, entre otros) (Zaika y col., 2001; Dang y col., 2006; Dang y col., 2008; Kress y col., 2015). La región TAD es necesaria para regular el crecimiento y ciclo celular, inhibición de diferenciación celular, regulación del metabolismo lo que sugiere las múltiples interacciones complejas que tiene c-Myc con otras proteínas celulares (Conacci-Sorrell y col., 2014; McMahon, 2014; Hann, 2014; Tu y col., 2015). Además, el dominio amino terminal de la proteína c-Myc posee varias regiones conservadas que se denominan cajas Myc (MBI, MBII, MBIII, MBIV), las cuales son esenciales para las funciones biológicas de esta proteína. MBI y MBII son responsables de la regulación de la transcripción y la transformación celular (Figura 6) (Flinn y col., 1998; Schwinkendorf y Gallant; 2009).

c-Myc sensibiliza a las células a sufrir apoptosis a través de diversos estímulos celulares. A continuación se describen los dos mecanismos más aceptados de inducción de apoptosis. En el primero, c-Myc induce la expresión de la proteína supresora de tumores p14ARF, la cual activa a p53 mediante la inactivación de la proteína E3 ubiquitina ligasa, HDM2. p53 activa a los genes proapoptóticos Bax y Puma. La proteína Bax se inserta en la membrana

mitocondrial favoreciendo la permeabilización de la membrana mitocondrial externa; este evento favorece la liberación de citocromo c y finalmente la inducción de la apoptosis (Hoffman y Liebermann, 2008; McMahon, 2014). En la segunda vía, la inducción de apoptosis por c-Myc implica la activación directa de la proteína proapoptótica Bax, la liberación de citocromo c y finalmente la activación de la cascada de caspasas que degradan los organelos celulares. c-Myc reprime la expresión de las proteínas Bcl-XL y Bcl-2 que regulan la expresión de Bax (Larsson y Henriksson, 2010; McMahon, 2014). Además, c-Myc, amplifica la actividad de caspasa-8 inducida por receptores de muerte (Figura 3) (Nieminen y col., 2007). En condiciones patológicas, la inhibición de la expresión de genes proapoptóticos como p53, Bax, Bim; o la sobreexpresión de genes antiapoptóticos como Bcl-2 y Bcl-XL, colaboran con el oncogén c-Myc y aceleran la tumorogénesis (McMahon, 2014; Ott, 2014).

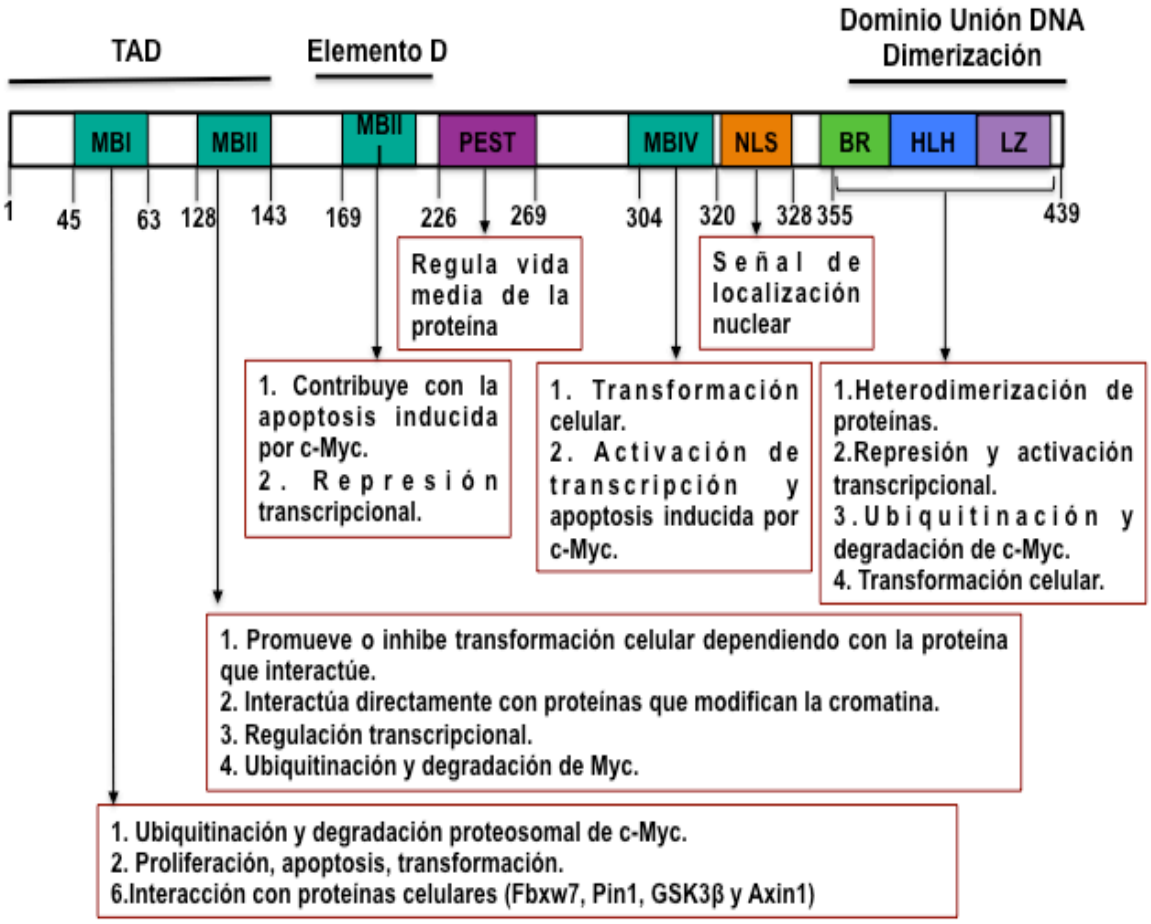


Figura 6. Estructura de la proteína c-Myc y funciones reguladas por sus dominios. En la región amino terminal se localiza la región de transactivación (TAD) y un elemento importante para proteólisis de la proteína (Elemento D) independiente de ubiquitinación. Además, se muestra un motivo proteico (PEST) presente en proteínas de vida corta. La proteína c-Myc presenta cuatro regiones conservadas conocidas como cajas c-Myc (MBI, MBII, MBIII, MBIV), las cuales son esenciales para las diferentes funciones de c-Myc. En la región carboxilo terminal se encuentran la región básica (BR) y la señal de localización nuclear (NLS); además de un dominio de dimerización HLH/LZ. Los cuadros representan las diversas funciones celulares que se realizan por cada dominio de la proteína.

4.7. Inhibición de apoptosis por E6- AR.

Las oncoproteínas E6 de alto riesgo (E6-AR) favorecen la degradación de diversas proteínas celulares. E6-AR inhiben la cascada de la vía extrínseca de la apoptosis a través de la unión o degradación de procaspasa-8, FADD, TNF-R1. De la misma forma, E6-AR participa en la inactivación de la vía intrínseca de la apoptosis a través de la degradación de p53, Bax, y Bak; e induce proteínas que inhiben la apoptosis como survivina y c-IAP-2. Al degradar E6 a p53 a través de la vía de la ubiquitina inhibe la activación transcripcional de genes blanco de p53 e impide la inhibición de Bcl-2 por p53 en la mitocondria. Además, E6 interactúa y degrada a dos proteínas reguladoras de la cromatina como son TIP60 y hADA3. Otra proteína que interacciona con E6 es ATF3, favoreciendo la liberación y activación de p53 del complejo E6-E6AP (Figura 7). E6 interacciona también con DFF40 inhibiendo la fragmentación del DNA (Lagunas-Martínez y col., 2010, Wang y col., 2010; Jha y col., 2010; Jong y col., 2012; Chand y col., 2014).

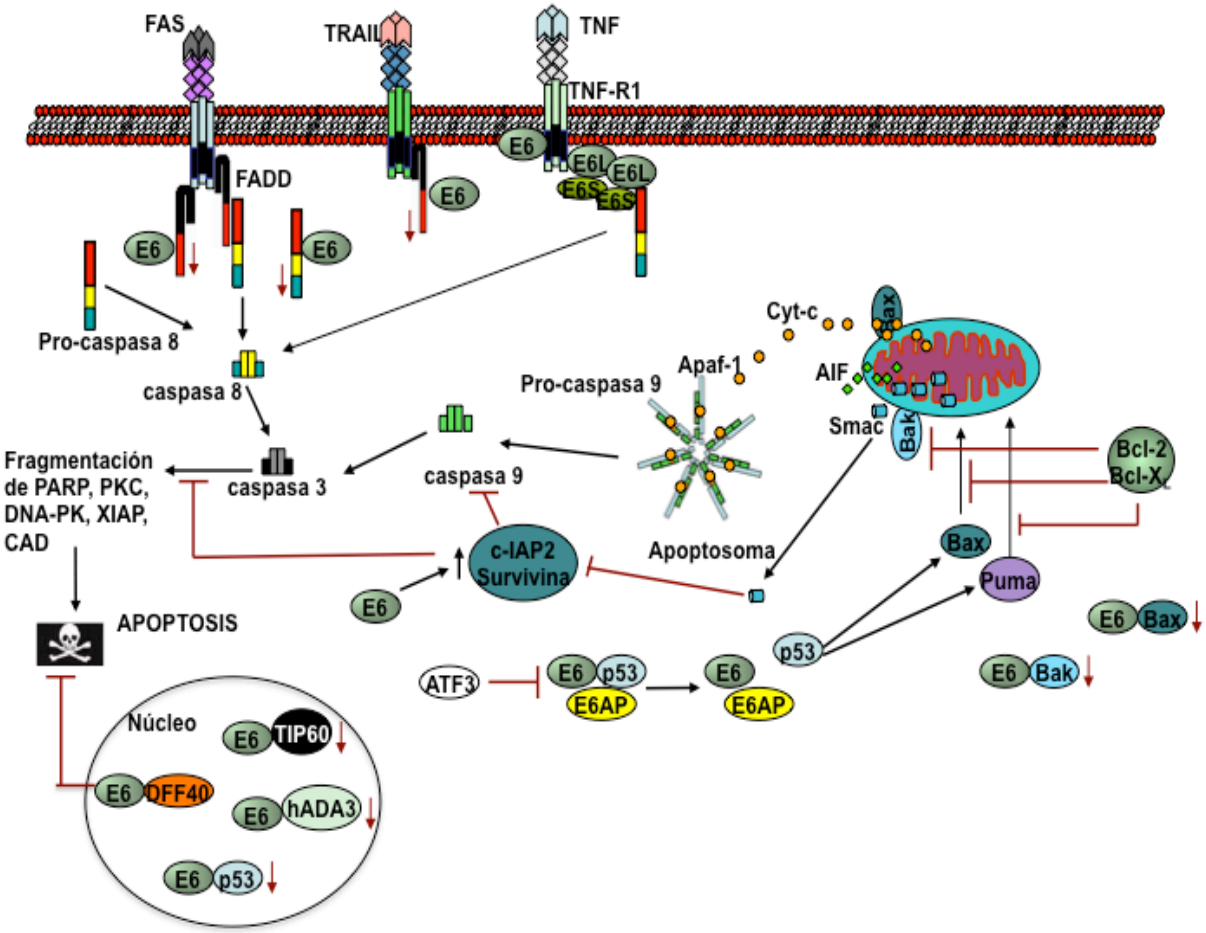


Figura 7. Regulación de la apoptosis por la oncoproteína E6-AR. En la imagen, se muestra las interacciones de la oncoproteína viral con proteínas celulares y su efecto sobre la apoptosis. La proteína E6 es frecuentemente asociada con inhibición de apoptosis debido a que favorece la degradación de proteínas proapoptóticas, como p53, Bax, and Bak. Además, la proteína viral E6 favorece la degradación/inactivación de proteínas que podrían regular la apoptosis (ATF3, hADA3, TIP60, DFF40) e inhibe la apoptosis inducida por receptores de muerte e induce la expresión de inhibidores de la apoptosis (IAP's, c-IAP2, survivina). La ↓ representa la degradación de proteínas celulares. E6L (E6 large), E6S (E6 small).

5. ANTECEDENTES ESPECÍFICOS

Durante la carcinogénesis del cuello uterino, una gran cantidad de proteínas que actúan como supresoras de tumores o proteínas proapoptóticas son inactivadas o degradadas permitiendo de esta forma la transformación celular. Diversos trabajos se han publicado en el que utilizan inhibidores del proteosoma para restablecer la expresión de estas proteínas (Aguilar-Lemarroy y col., 2002; Bossi y Sacchi, 2007; Harris y col., 2008). Estos inhibidores se han utilizado para evitar la degradación de p53 en células que expresan a la oncoproteína E6-AR, la cual actúa como una E3 ubiquitina ligasa. En un trabajo previo (Aguilar-Lemarroy y col., 2002), fue reportado que queratinocitos inmortalizados con E6 del VPH16 fueron insensibles a la acción de una molécula que mimetiza la acción de CD95L, mientras queratinocitos inmortalizados con E7 fueron sensibles a la acción del ligando. Los queratinocitos E6 del VPH16 (QE6-16) fueron sensibilizados a apoptosis inducida por el ligando posterior a la inhibición del proteosoma 26S a través de MG132. Posterior al tratamiento con MG132 se restablecieron los niveles de la proteína p53 y c-Myc (Aguilar-Lemarroy y col., 2002). La activación de la proteína p53 ha sido analizada como una estategía terapeutica en diversos tipos de neoplasias (Bossi y Sacchi, 2007; Harris y col., 2008), y en CaCU no ha sido la excepción. La proteína 53 ha sido sugerida como molecula potencial para inducer apoptosis posterior su rescate de degradación por MG132 (Aguilar-Lemarroy y col., 2002; Hougardy y col., 2006). Por lo anterior, en el presente trabajo de tesis se evaluó el papel que tiene p53 en la inducción de apoptosis inducida por el anticuerpo APO-1, el cual mimetiza la acción de CD95L.

6. JUSTIFICACIÓN

El CaCU es un problema de salud pública a nivel mundial. El virus del papiloma humano de alto riesgo (VPH-AR) es el agente etiológico del CaCU. Debido a la expresión de las oncoproteínas virales E6 y E7 en las células infectadas por el VPH-AR, una plétora de proteínas celulares son inactivadas. La oncoproteína E6 favorece principalmente la inactivación de proteínas proapotóticas (caspasa-8, FADD, TNF-R1) a través del complejo del proteosoma 26S inhibiendo la activación de la vía extrínseca. Además, uno de los principales blancos de inactivación y degradación de E6 es la proteína supresora de tumores p53, favoreciendo la proliferación sin control e inhibiendo la activación de muerte celular.

En CaCU, tratamientos que impliquen la activación de la vía extrínseca de la apoptosis a través de receptores de muerte son inhibidos debido a la actividad de E6, además del proceso de transformación e inmortalización celular en el cual se inhibe la expresión de múltiples genes supresores de tumores.

Por lo anterior, es importante determinar a través de que mecanismo las células tumorales o transformadas, pueden sensibilizarse a apoptosis. Debido a que queratinocitos humanos inmortalizados con E6 (QE6-16) se vuelven sensibles a apoptosis por la vía CD95 posterior a la estabilización de p53 y c-Myc por el inhibidor del proteosoma MG132; en este proyecto se pretende caracterizar el papel de p53 en dicho evento.

7. HIPÓTESIS

La activación de la vía de CD95 en queratinocitos humanos inmortalizados con E6 del VPH16 estimulados con MG132, sensibiliza a las células a muerte celular a través de la estabilización de p53.

8. OBJETIVO GENERAL

Determinar la participación de p53 en la sensibilización de la muerte celular generada por la activación de la vía de CD95 y MG132 en queratinocitos humanos inmortalizados con E6 del VPH 16.

9. OBJETIVOS ESPECÍFICOS

Evaluar la inducción de apoptosis a través de la activación de la vía CD95 y MG132 en QE6-16.

Evaluar la inducción de autofagia a través de la activación de la vía CD95 y MG132 en QE6-16.

Determinar la estabilidad de la proteína p53 y su fosforilación en Ser20 y Ser46 en QE6-16 sensibilizados a apoptosis a través de la activación de la vía CD95 y MG132.

Determinar la localización intracelular de la proteína p53 en QE6-16 sensibilizados a apoptosis a través de la activación de la vía CD95 y MG132.

Determinar la inducción de genes blanco proapoptóticos de p53 en QE6-16 sensibilizados a apoptosis a través de la vía CD95 y MG132.

Determinar el papel de p53 y c-Myc en QE6-16 sensibilizados a apoptosis a través de la activación de la vía CD95 y MG132.

10. METODOLOGÍA

10.1. Cultivo celular

Queratinocitos humanos inmortalizados con E6 de VPH16 (QE6-16) fueron proporcionados por el grupo del Dr. Frank Rösl (Heidelberg, Germany) y han sido previamente reportados (Aguilar-Lemarroy y col., 2002). QE6-16 fueron cultivados en medio para queratinocitos (K-SFM), suplementado con 20 μg/ml de extracto de glándula pituitaria bovina, 0.1 ng/ml del factor recombinante de crecimiento epidermal (Gibco ®), 2% de suero fetal bovino y una mezcla de antibiótico-antimicótico (Invitrogen^{TM}) en una atmosfera de 5% de CO_2 a 37°C. Para los diferentes experimentos que se realizaron, QE6-16 fueron incubados en presencia de DMSO (control), MG132 (20 μM), APO-1 (100 ng/ml) o la combinación de ambos tratamientos (MG132 más APO-1) en los tiempos indicados.

10.2. Ensayo de Apoptosis (Anexina)

QE6-16 fueron cultivados en placas de 6 pozos ($8x10^5$ células/pozo) e incubados 24 h con los diferentes tratamientos. Las células fueron lavadas con PBS, desprendidas y colectadas por centrifugación. Posteriormente, las células fueron resuspendidas en 100 μl de buffer de unión a Anexina, adicionando 5 μl de Anexina V-FITC y 50 μg/ml de ioduro de propidio (IP) (BioVision). Las células fueron incubadas a temperatura ambiente por 10 min. en condiciones de oscuridad, posteriormente las células fueron centrifugadas a 2500 rpm y lavadas con PBS 1X. Las células fueron analizadas en el citómetro de flujo FACS Scalibur (Becton Dickinson, San José, CA, USA). Células que presentaron translocación de fosfatidilserina hacia la membrana externa fueron identificadas utilizando la señal del detector FITC (FL1). Células IP positivas fueron detectadas a través del detector de señal de emisión para ficoeritrina (FL2). El análisis fue realizado en 10,000 eventos celulares utilizando el software Summit V4.3.

10.3. Ensayo de permeabilidad de la membrana mitocondrial

Las células fueron cultivadas y tratadas como previamente fue descrito en el ensayo de Anexina. Después del tratamiento por 24 h, las células fueron desprendidas e incubadas en la presencia de 200 nM de TMRE por 15 min a 37°C. Las células fueron lavadas con PBS y la intensidad de fluorescencia fue analizada a través del citómetro de flujo FACS Scalibur utilizando el detector de señal FL2. El análisis fue realizado sobre 10,000 eventos celulares utilizando el software Summit V4.3.

10.4. Ensayo de permeabilidad de la membrana lisosomal

Las células fueron sembradas y tratadas como fue descrito en los anteriores ensayos de citometría. Brevemente, las células fueron desprendidas e incubadas con 5 μg/ml de naranja de acridina (NA) por 15 min a 37°C. QE6-16 fueron lavados con PBS y 10,000 células fueron analizadas utilizando el citómetro de flujo FACS Scalibur, utilizando la señal de detección FL3. El análisis fue realizado utilizando el software Summit V4.3.

10.5. Ensayo de actividad de caspasas-3/7, 8 y 9

Se sembraron 10,000 células por pozo en una placa de 96 pozos en medio para queratinocitos (K-SFM). Posterior a las 24 h de tratamiento se determinó la actividad de las caspasas iniciadoras 8, 9 y las capasas ejecutoras 3/7, agregando 100 μl del reactivo Caspase-Glo a cada pozo (Promega, Madison, WI). Posteriormente, la placa de cultivo fue incubada por 30 min a temperatura ambiente, al término de este período la placa fue agitada a 300 rpm por 30 s. Una reacción blanco fue incluida, la cual contiene solo medio de cultivo y reactivo Caspase-Glo (sin células). La luminiscencia de cada muestra fue medida en un luminómetro (Glomax, Promega, Madison WI).

10.6. RT-PCR

Posterior a los diferentes tratamientos de 800,000 mil células sembradas en cajas de 6 pozos, se aisló RNA total utilizando el reactivo Trizol (Invitrogen). Su integridad fue

determinada por electroforesis en geles de agarosa al 1%. La concentración del RNA y su pureza (260/280) fue evaluada utilizando el espectrofotómetro NanoDrop LITE (Thermo Scientific). La cadena complementaria del DNA (cDNA) fue sintetizada utilizando 1 ug de RNA total y 200 U de la reverso transcriptasa M-MLV (Invitrogen) en presencia de 0.5 μg de oligo-dT (Invitrogen) en condiciones estándar. Las reacciones de PCR fueron realizadas en un volumen final de 25 ul que contiene 1 ul de cDNA, 2.5 mM de dNTP Mix, 1 U de Taq DNA polimerasa, 1X de Buffer PCR, 2.5 mM de $MgCl_2$, y 10 pMol de cada uno de los primers. Todos los reactivos fueron obtenidos de Invitrogen. La expresión del RNA mensajero de E6 de HPV16 fue determinada utilizando los primers mostrados en la Tabla 1. Las amplificaciones de la PCR se realizaron en el equipo Mastercycler PCR gradient (Eppendorf, Germany) utilizando las siguientes condiciones: 5 min a 94 °C; 35 ciclos a 94 °C por 1 min, 60 °C por 1 min, y 72 °C por 1 min**,** y una extensión final a 72 °C por 10 min. Se realizó una electroforesis en geles de poliacrilamida al 6 % para visualizar los productos amplificados por PCR. Los geles fueron teñidos con bromuro de etidio y analizados en el transiluminador (UV Transilluminator, UVP). Los primers para amplificar un fragmento del gen humano gliceraldehído-3-fosfato deshidrogenasa (GAPDH) fueron diseñados utilizando el software Primer Express V3.0 (Applied Biosystems) (Tabla 1).

10.7. qPCR

Para realizar el PCR cuantitativo (qPCR), se utilizó el cDNA previamente generado. Para realizar los ensayos se realizó una dilución 1:10 del cDNA en agua grado biología molecular. El qPCR fue realizado utilizando 2 ul de cada uno de los cDNAs diluidos y 1X de SYBR Green PCR Master Mix (Applied Biosystems) de acuerdo al protocolo proporcionado por el proveedor. La amplificación de genes blanco de p53 fue realizada utilizando los primers cuyas secuencias se muestran en la Tabla 1. Los primers de Hipoxantina Guanina Fosforribosil Transferasa fueron obtenidos de Qiagen y fueron utilizados para normalizar la expresión del RNA mensajero (RNAm) de p21, TP53INP1, Bax y LC3B en cada una de las muestras. El programa de PCR fue realizado bajos las siguientes condiciones: 10 min a 95°C; 40 ciclos de 15 s a 95°C y 1 min a 60°C. La

especificidad de la amplificación de los productos y la ausencia de dímeros de primers fue determinada a través de análisis de curvas de disociación.

PCR	Primer	Secuencia (5´→3´)	Referencia
Tiempo Real	TP53INP1-S	GCACCCTTCAGTCTTTTCCTGTT	Tomasini y col., 2005
	TP53INP1-AS	GGAGAAAGCAGGAATCACTTGTATC	
	Bax-S	GGGGACGAACTGGACAGTAA	Petre y col., 2007
	Bax-AS	CAGTTGAAGTTGCCGTCAGA	
	p21-S	GGAAGACCATGTGGA CCTGT	Petre y col., 2007
	p21-AS	GGC GTT TGG AGT GGT AGA AA	
	LC3B-S	AGGGTAAACGGGCTGTGTGA	Primer Express V3.0 software,
	LC3B-AS	CCCCTGCAAGAGTGAGGACTT	
Punto Final	GAPDH-S	ACCACAGTCCATGCCATCAC	
	GAPDH-AS	TCCACCACCCTGTTGCTGTA	
	E6 VPH16-S	ACTGCAATGTTTCAG GACCC	Aguilar-Lemarroy y col., 2002
	E6 VPH16-AS	TCAGGACACAGTGGCTTTTG	

Tabla 1. Secuencia de primer utilizados para PCR en tiempo real.

La curva estándar para cada gen fue generada a través de 5 diluciones seriadas del cDNA obtenido de QE6 tratados con MG132 (20 μM). La eficiencia de la amplificación de PCR para cada gen fue calculada utilizando el método de la curva estándar $E=10^{(-1/slope)}-1$. La expresión relativa fue calculada utilizando el método comparativo del ciclo umbral (CT) ($2^{-\Delta\Delta CT}$) (Livak y Schmittgen, 2001).

10.8. Western blot

Después del tratamiento, QE6-16 ($8x10^5$ células por pozo) fueron lavados y lisados con buffer RIPA suplementado con inhibidores de proteasas y fosfatasas. Los extractos proteicos fueron incubados durante 30 min en hielo con agitación en vortex constante para una completa disgregación de las células. Posteriormente, la muestra fue centrifugada a 12,000 rpm por 30 min. La concentración de la proteína fue determinada utilizando el kit BCA Protein Assay (Thermo Scientific, Rockford, IL). Cincuenta μg de proteína total fueron separados a través de geles de poliacrilamida SDS al 10 o al 15% (según fuera el caso) en condiciones desnaturalizantes y transferidas a membrana de nitrocelulosa (GE

Healthcare, Buckinghamshire, UK). Las membranas fueron bloqueadas por una hora con 5% de leche libre de grasa *Marca) e incubada con el anticuerpo anti-p53-HRP, actina-HRP, LC3B, Bid toda la noche a 4°C. Para la detección de la proteína p53 fosforilada en Ser20 o Ser46, la membrana fue bloqueada con 5% de BSA. Posteriormente, las membranas fueron lavadas tres veces con PBS/0.1% Tween-20. Para los anticuerpos primarios que no estaban marcados con HRP, se requirió una segunda incubación con un anticuerpo anti-conejo HRP. Las proteínas fueron visualizadas utilizando el kit SuperSignal West Pico chemiluminiscence (Pierce, Thermo Scientific, Rockford, Illinois, USA) y la señal fue adquirida a través de películas de rayos-X (Kodak, St. Louis MO, USA). El anticuerpo anti-actina-HRP fue utilizado como un control de carga. El análisis densitométrico de las bandas correspondientes a la expresión de p53 y actina fue realizado utilizando el programa Image J.

10.9. Microscopia confocal

QE6-16 ($5x10^4$ células por pozo) fueron crecidas sobre placas de 8 pozos Lab-Tek (Nalge Nunc International, Roskilde, Denmark). Después del tratamiento, las células fueron lavadas con PBS estéril y fijadas con parafolmaldehído al 2% en PBS 1X por 10 min a 4°C. Las células fueron lavadas nuevamente con PBS 1X y bloqueadas con CAS-Block (Zymed) por 15 min a 37°C. Después de remover el CAS-Block, los pozos fueron lavados con PBS 1X. Las células fueron incubadas toda la noche a 4°C con el anticuerpo anti-p53 (ratón) y entonces lavados con PBS 1X. Posteriormente, las células fueron incubadas en oscuridad por 2 horas a 37°C con un anticuerpo anti-ratón marcado con Alexa Fluor® 488 para la detección de p53. Para la tinción de núcleos, las células fueron incubadas por 15 min con 100 ng/ml de DAPI en PBS 1X. Las células fueron lavadas con PBS 1X y preparadas con medio permanente de montaje. Finalmente, las células fueron analizadas utilizando el microscopio confocal Leica DMI 4000B utilizando el objetivo de inmersión 63X. Para la detección de los fluoróforos Alexa® 488/DAPI, se utilizó el láser de excitación verde-azul. Las imágenes fueron analizadas utilizando el programa LAS AF Lite. Se contaron 100 células por pozo en los diferentes tratamientos y tiempos para detectar la señal de p53 nuclear. Cada tratamiento fue realizado por triplicado.

10.10. Ensayo de citotoxicidad

Células HeLa ($1x10^4$), SiHa ($1x10^4$), QE6-16 ($1x10^4$) o cultivo primario de queratinocitos humanos (QN) ($1x10^4$) fueron cultivados en caja de 96 pozos. Posterior a las 24 h de los diversos tratamientos, se agregaron 20 ul de MTS (Promega). La absorbancia fue directamente proporcional al porcentaje de células viables. Una reacción blanco fue incluida, la cual contiene solo medio de cultivo y reactivo MTS (sin células). La absorbancia fue determinada en un lector de placas de ELISA (Lab Systems multiskan MS).

10.11. Análisis estadístico

Los datos son presentados como la media ± desviación estándar (DS). El análisis estadístico fue realizado utilizando la prueba Wilcoxon-Mann-Whitney. Diferencias de $p<0.05$ fueron consideradas estadísticamente significativas. Todos los análisis fueron realizados utilizando la versión 12 del software STATA (StataCorp, Collage Station, TX, EUA).

11. RESULTADOS

11.1. APO-1 y MG132 induce apoptosis en una línea celular de queratinocitos humanos que expresan la oncoproteína E6 (QE6-16)

Para el desarrollo de este trabajo se utilizaron queratinocitos humanos inmortalizados con el oncogén E6 del VPH-16, los cuales fueron proporcionados por el grupo del Dr. Frank Rösl y han sido previamente descritos (Aguilar-Lemarroy, 2002). Antes de iniciar la estrategia experimental, se evaluó la expresión del RNA mensajero del oncogén E6 a través de la técnica de RT-PCR. Utilizando primers específicos para E6 del VPH16 se observó la amplificación de 2 productos, uno de 344 pb (E6), y otro de 161 pb (E6*) (Figura 8).

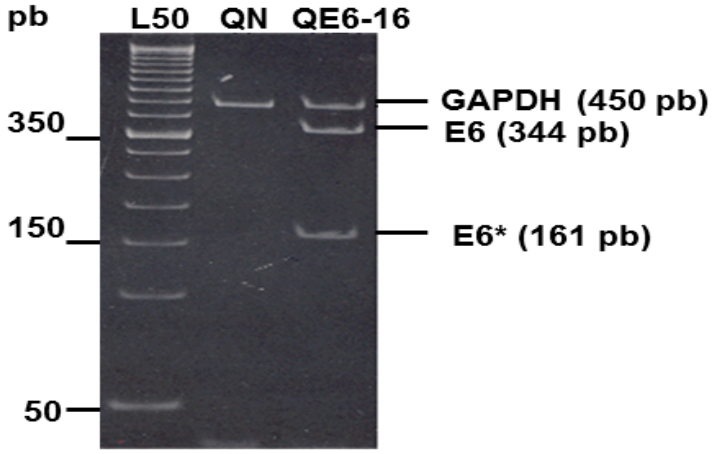


Figura 8. Expresión del oncogén viral E6 de VPH16 en queratinocitos humanos inmortalizados (QE6-16). Se extrajo RNA total de un cultivo primario de queratinocitos normales (QN) y de QE6-16. Los productos de PCR generados para E6-16 y GAPDH fueron separados en geles de acrilamida al 6%. Los productos amplificados de E6 y E6* (de 344 y 161 pb, respectivamente) son indicados. L50: Marcador de 50 pb.

Posteriormente, se incubaron las células en presencia de 100 ng/ml de APO-1 (un activador de la vía extrínseca de la apoptosis), 20 μM de MG132 (un inhibidor del proteosoma 26S), o ambos por 24 h. La apoptosis fue determinada a través de citometría de flujo utilizando el

ensayo de Anexina V/Ioduro de propidio (IP). Los resultados obtenidos mostraron un incremento en la apoptosis del 47% después de la inactivación del proteosoma por MG132 y activación de la vía extrínseca de la apoptosis mediada por el anticuerpo APO-1 (APO-1 más MG132) (Figura 9A y B). Sin embargo, el sólo tratamiento con MG132 también mostro una elevada inducción de la apoptosis del 29%, mientras el tratamiento con APO-1 mostro un incremento del 6 %. Bajos niveles de apoptosis (2.9%) fueron observados en el grupo control (DMSO). Los tratamientos con APO-1, MG312, y APO-1 más MG132 fueron estadísticamente significativos al compararlos con el control ($p<0.05$).

Para identificar las vías de la apoptosis activadas por MG132 y APO-1, se analizó la activación de la vía intrínseca y extrínseca a través de la activación de caspasa-9 y 8, respectivamente. Después del tratamiento de QE6-16 con 100 ng/ml de APO-1, 20 μM de MG132 o la combinación de ambos por 24 h, se encontró que ambas vías (Figura 9C y D) fueron activadas por los tratamientos individuales (APO-1 o MG132) o combinados (APO-1 más MG132) ($p<0.05$). Sin embargo, el tratamiento combinado mostró una mayor activación de caspasa-8 o 9 que en los grupos individuales y que en el grupo control. Aunque se ha reportado que la oncoproteína viral E6 favorece la degradación de proteínas involucradas en la activación de receptores de muerte, también se encontró la activación de la vía extrínseca en las células tratadas solamente con APO-1 (Figura 9C). Este resultado sugiere que en la vía extrínseca, la apoptosis es inicialmente activada a través de caspasa-8, la cual fragmenta a la proteína Bid generando Bid truncada (tBid), una proteína proapoptótica que favorece la liberación de citocromo-c (Cyt-c), la activación de caspasa-9 y la vía intrínseca de la apoptosis.

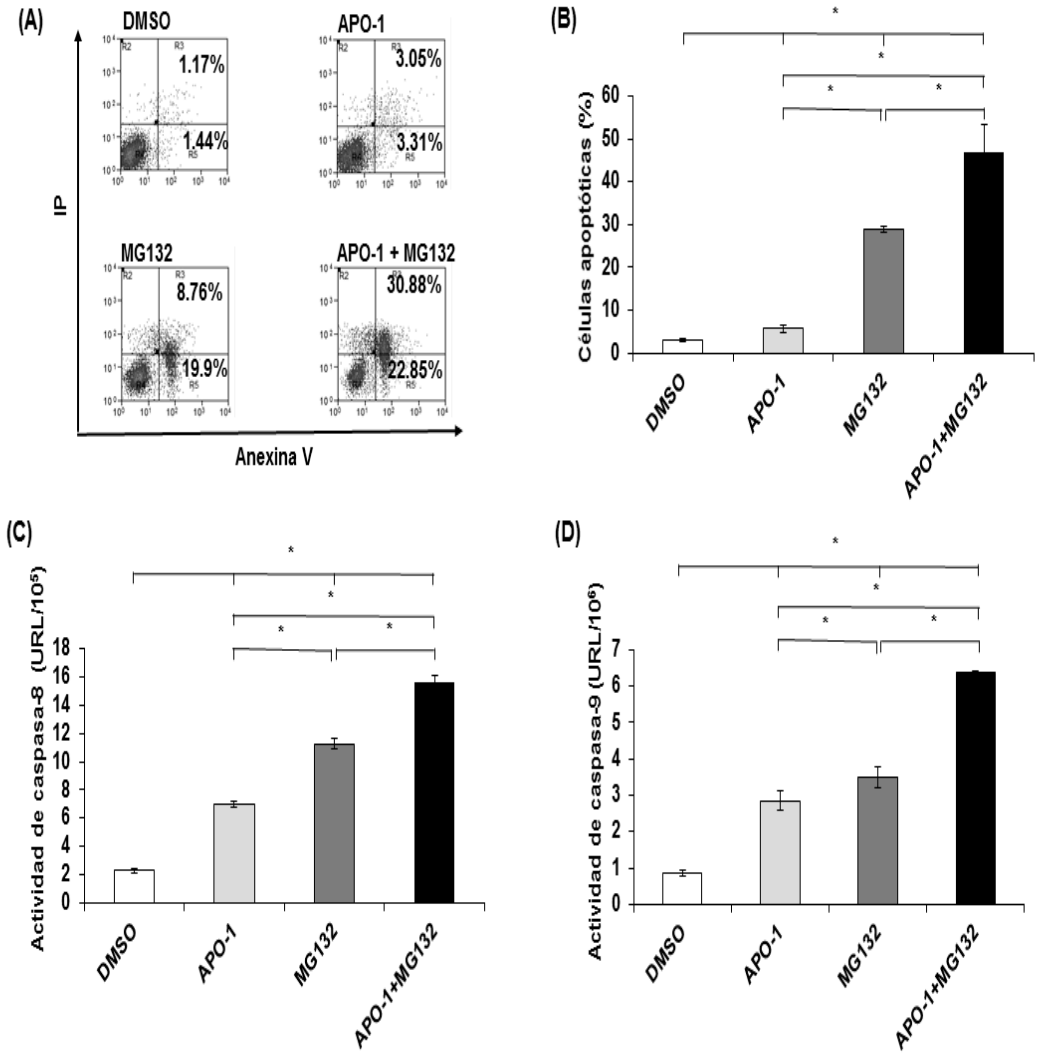


Figura 9. Inducción de la apoptosis en QE6-16 incubados con APO-1 y MG132 por 24 h. Las células se incubaron con 20 μM de MG132 (inhibidor del proteosoma), 100 ng/ml de APO-1 (anticuerpo que mimetiza la acción del ligando CD95 e induce la respuesta apoptótica), o ambos compuestos por 24 h. Posteriormente, las células fueron teñidas con una combinación de Ioduro de propidio (IP) y Anexina V para detectar células apoptóticas. **(A)** Histograma representativo de citometría de flujo de células apoptóticas (utilizando Anexina V). **(B)** Cuantificación de la intensidad de fluorescencia que fue medida por citometría de flujo usando los canales FL1 (Anexina V) y FL2 (PI). La actividad de caspasa-8 **(C)** y caspasa-9 **(D)** se determinó por hidrólisis del substrato luminogénico que contiene la secuencia DEVD (secuencia reconocida por las caspasas). Las lecturas fueron determinadas a la 0.5 h después de adicionar el substrato de la caspasa. Bajo estas condiciones, la luminiscencia es proporcional a la actividad de la caspasa-8 y 9 expresada como Unidades Relativas de Luz (URL). El valor obtenido del control (grupo sin células) fue restado de cada determinación de URL. Las gráficas representan la media ±DS de tres ensayos independientes (**p<0.05*). El análisis estadístico fue realizado entre cada grupo de tratamiento con el control (DMSO) o entre los diferentes grupos.

11.2. APO-1 más MG132 favorecen la activación de la vía mitocondrial de la apoptosis (intrínseca) en QE6-16

Ha sido demostrado que cambios en el potencial de la membrana mitocondrial favorecen la liberación de citocromo c de la membrana interna de la mitocondria al citoplasma favoreciendo la apoptosis. Para determinar la participación de la mitocondria (vía intrínseca de la apoptosis) en la inducción de la apoptosis mediada por APO-1 y MG132, se analizó la pérdida del potencial de la membrana mitocondrial ($\Delta\Psi m$) a través de citometría de flujo utilizando el colorante catiónico tetrametil rodamina metil éster. Los resultados mostraron una importante reducción en el potencial de membrana con el tratamiento combinado (APO-1 más MG132), sugiriendo la liberación de Cyt-c y la inducción de la apoptosis. Además, el tratamiento con MG132 mostró un alto porcentaje en la reducción del potencial de membrana (22.8%). Un modesto pero significativo cambio en el potencial de membrana fue observado después del tratamiento con APO-1 (Figura 10A y B). Similar a la apoptosis, el ensayo de TMRE mostró un efecto aditivo en el tratamiento de APO-1 más MG132 ($p<0.05$). Para identificar la posible fragmentación de Bid (tBid, proteína proapoptótica que favorece la liberación de Cyt-c, y activación de la vía intrínseca de la apoptosis generada por la activación de CD95), se evaluó la fragmentación de esta proteína a través de Western Blot. Los resultados mostraron una disminución en los niveles de expresión de la proteína Bid (22 kDa) en los QE6-16 tratados con APO-1 más MG132 a las 12 y 24 h (Figura 10C), sugiriendo que la activación de la vía intrínseca de la apoptosis requiere la activación de Bid.

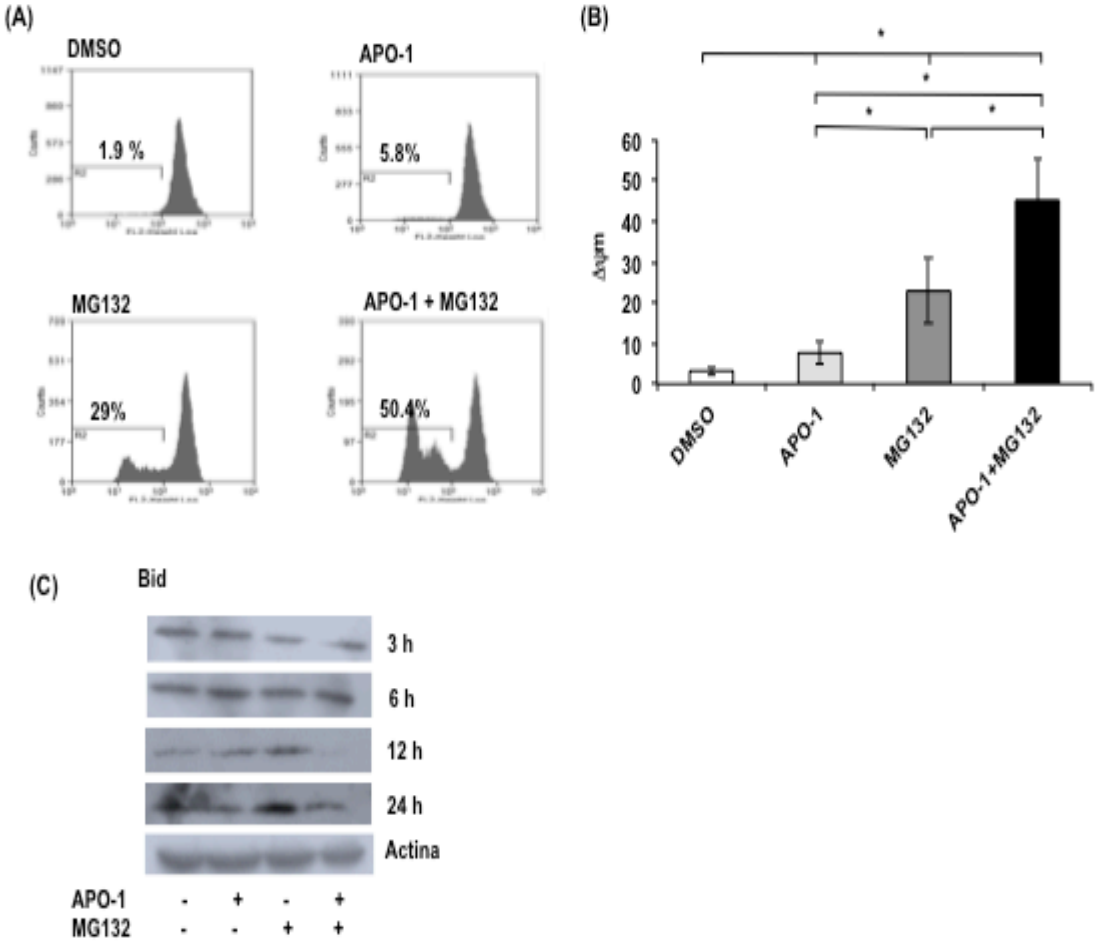


Figura 10. Efecto de APO-1 y MG132 en el potencial de membrana mitocondrial (Δψm) de QE6-16. **(A)** Histograma representativo de QE6-16 cultivados en presencia de 20 μM de MG132, 100 ng/ml de APO-1, o ambos tratamientos durante 24 h. **(B)** Posterior a 24 h de tratamiento las células fueron teñidas con el colorante fluorescente TMRE. Las gráficas representan la media ±DS de tres ensayos independientes. El asterisco representa resultados estadísticamente significativos con respecto al control (DMSO) (**p<0.05*) o entre los diferentes grupos. **(C)** Western blot de Bid usando extractos totales de proteínas de QE6-16 (50 μg de proteína/carril) tratados por 3, 6, 12 y 24 h con 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos. La detección de actina fue utilizada como control de carga. La electroforesis fue realizada en geles de poliacrilamida al 15%. Los resultados son representativos de tres experimentos independientes.

11.3. APO-1 y MG132 inducen autofagia en QE6-16

Para identificar si la autofagia es activada en QE6-16 cultivados en presencia de APO-1 y MG132, se evaluó la permeabilidad de la membrana lisosomal a través de citometría de flujo, analizando la retención de Naranja de Acridina (AO, por sus siglas en inglés) en los lisosomas. Los resultados mostraron un incremento estadísticamente significativo en la permeabilidad lisosomal en QE6-16 tratados con MG132 o APO-1 más MG132 (Figura 11A y B)(p<0.05). En contraste, en las células tratadas con APO-1 y el grupo control se observó un ligero incremento en la permeabilidad lisosomal (Figura 11B), sugiriendo que el tratamiento con MG132 es el principal activador de la autofagía. La activación de la autofagía fue confirmada por la detección de la forma lipidada de la proteína LC3B a través de Western blot en células tratadas con MG132 y APO-1 más MG132 (Figura 11C).

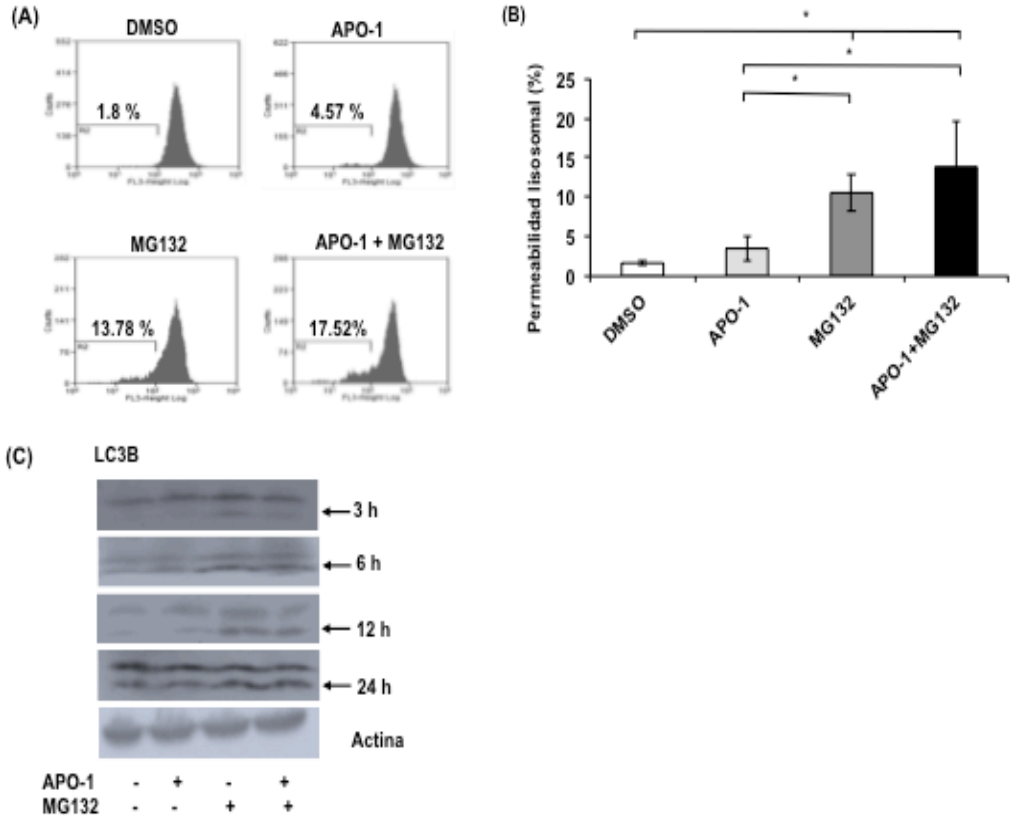


Figura 11. Efecto de APO-1 y MG132 en la integridad de la membrana lisosomal y lipidación de LC3B en QE6-16. **(A)** Histogramas representativos de QE6-16 cultivados durante 24 h en presencia de 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos. **(B)** Análisis de citometría de flujo realizado en QE6-16 incubados con 20 μM de MG132,

100 ng/ml de APO-1 o ambos compuestos durante 24 h. Posterior al tratamiento, las células fueron teñidas con Naranja de acridina (NA). Los valores indican el porcentaje de células que presentaron una baja fluorescencia de NA (alta permeabilidad lisosomal). Las gráficas representan el promedio ±DS de tres experimentos independientes. Las diferencias entre los grupos fueron estadísticamente significativos cuando *$p<0.05$. **(C)** La detección (a través de Western blot) de la forma lipidada de la proteína LC3B (50 µg de proteína/carril) se indica con una flecha. La electroforesis fue realizada en geles de poliacrilamida al 15 %.

11.4. El tratamiento con APO-1 más MG132 estabiliza a la proteína p53

Se ha demostrado que la oncoproteína E6 induce una fuerte reducción de los niveles de la proteína p53 a través de la vía de la ubiquitina (Scheffner y col., 1990); y que el inhibidor del proteosoma MG132, restablece los niveles de la proteína p53 (Aguilar-Lemarroy y col., 2002). Debido a que los efectos de APO-1 y APO-1 más MG132 sobre los niveles de la proteína p53 son desconocidos, se analizó la expresión de esta proteína a través de Western blot a las 3, 6, 12 y 24 h en QE6-16. Los resultados obtenidos mostraron que el tratamiento con MG132 y APO-1 más MG132 restablecen los niveles de p53 a las 3, 6 y 12 h (Figura 12A-C). La detección de p53 a las 24 h fue prácticamente indetectable, probablemente como una consecuencia de la degradación de proteínas celulares durante la apoptosis.

Se ha reportado que varias cinasas se unen y fosforilan a p53 en diversas regiones como consecuencia de estrés celular. Para determinar si p53 es fosforilado en Ser20 y Ser46 (la cual es una modificación postraduccional clave en la inducción de apoptosis) (Hofmann y col., 2002), se extrajeron las proteínas de células tratadas con APO-1, MG132 o la combinación de ambos compuestos y se evaluó la fosforilación a través de Western blot. Los resultados mostraron fosforilación en p53 en Ser20 y Ser46 a las 6 h (Figura 12D). Interesantemente, se observó una débil banda en las células tratadas con APO-1 comparadas con las células tratadas con MG132 y APO-1 más MG132. Este hallazgo fue inesperado y en este momento se desconoce su importancia biológica.

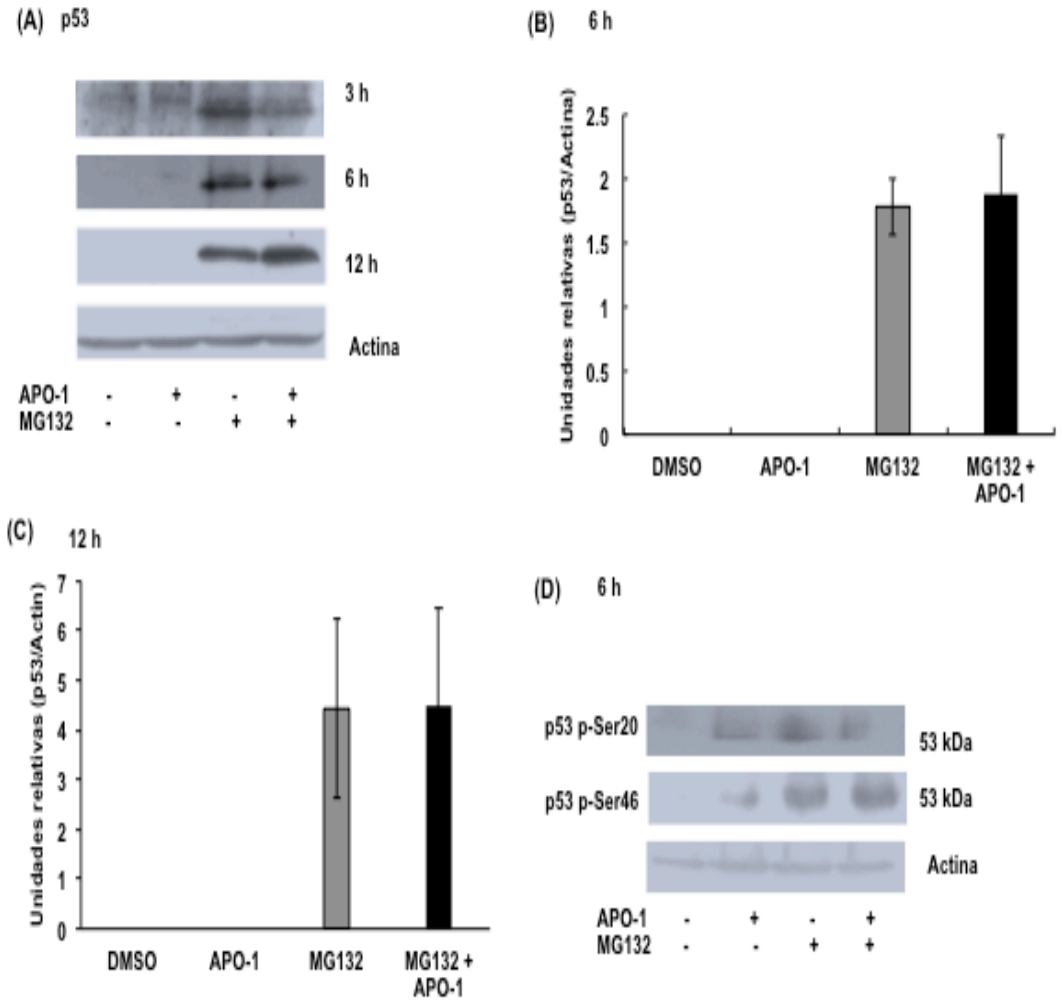
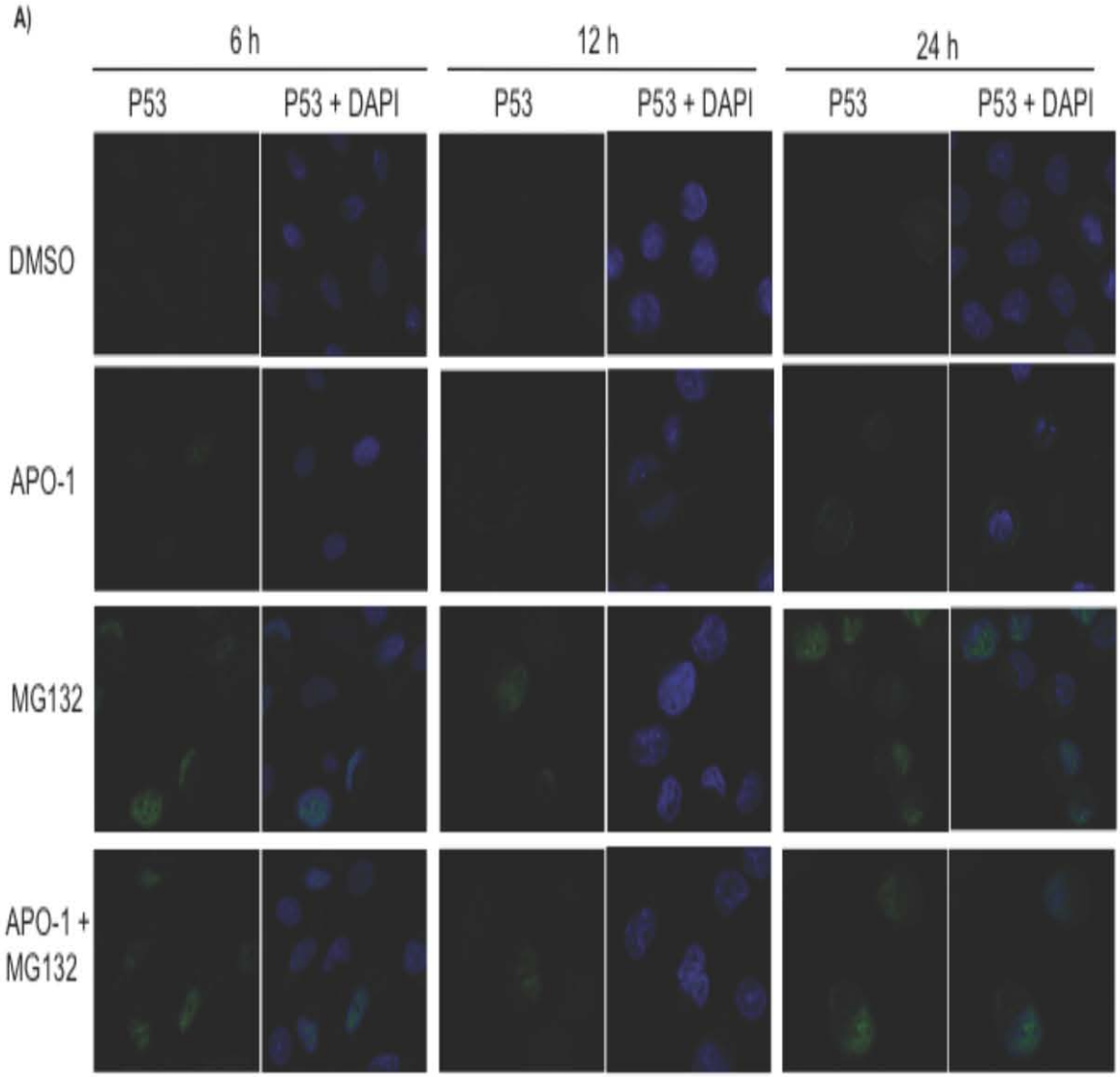


Figura 12. La inhibición del proteosoma restablece los niveles de la proteína p53, la cual presenta fosforilación en Ser20 y Ser46 en QE6-16. **(A)** Imágenes representativas de la inmunodetección de p53 utilizando 50 μg de proteína/carril de QE6-16 cultivados en presencia de 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos por un período de 3, 6 y 12 h. Los niveles de expresión de actina fueron utilizados como control de carga. La electroforesis se realizó en geles de poliacrilamida al 10%. Los datos mostrados son representativos de tres experimentos independientes. **(B, C)** Las gráficas representan el promedio del análisis de densitometría de p53. **(D)** Imágenes representativas de Western blot de las fosforilaciones de p53 en Ser20 y Ser46 utilizando 50 μg de proteína total por carril. Los QE6-16 fueron cultivados en presencia de 20 μM de MG132, 100 ng/ml APO-1 o ambos compuestos durante 6 h.

11.5. Durante la apoptosis inducida por APO-1 más MG132 en QE6-16 p53 es acumulada en el núcleo

Diversas evidencias indican que la proteína p53 puede modular la apoptosis y autofagia dependiendo de sus modificaciones postraduccionales y su localización celular (Tang y col., 2015). P53 es un factor de transcripción nuclear que activa la expresión de genes proapoptóticos y proautofágicos, o que inhiben el ciclo celular. Además, p53 promueve la apoptosis e inhibe la autofagia cuando se localiza en la mitocondria, o induce la activación de ambos tipos de muerte cuando se encuentra en el núcleo (Tang y col., 2015). Por lo anterior, en este trabajo se determinó la localización celular de p53.

QE6-16 fueron cultivados en presencia de APO-1, MG132, o la combinación de ambos compuestos durante 6, 12 y 24 h. Los resultados de microscopía confocal mostraron que células tratadas con MG132 o APO-1 más MG132 presentaban una fuerte señal de p53 en el núcleo. Una débil señal de la proteína p53 fue detectada en el citoplasma de células tratadas con APO-1 (y en algunos casos en el núcleo). En unos pocos casos la señal de p53 nuclear fue detectada en células control (Figura 13A). En los diferentes tiempos analizados, la señal de p53 en el núcleo se observó principalmente en las células cultivadas en presencia de MG132 y APO-1 más MG132 (Figura 13B). Debido a la gran cantidad de células muertas y desprendidas a las 24 h con el tratamiento APO-1 más MG132, solo se contó aproximadamente el 40% del total de las células, en las cuales en todas las células p53 se localizó en el núcleo. Estos resultados sugieren que p53 debería de inducir muerte celular a través de la inducción transcripcional de sus genes blanco en las células cultivadas en presencia de MG132 y APO-1 más MG132.


A)
6 h
12 h
24 h
P53
P53 + DAPI
P53
P53 + DAPI
P53
P53 + DAPI
DMSO
APO-1
MG132
APO-1 +
MG132

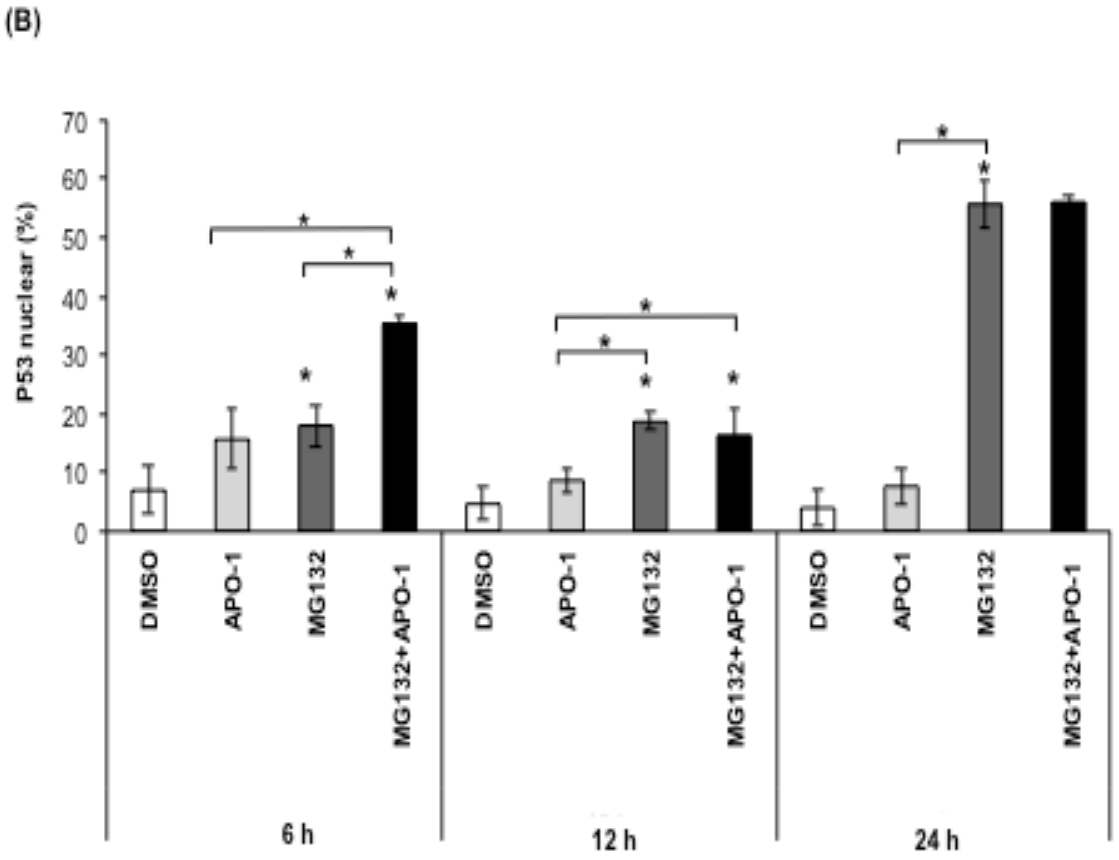


Figura 13. Los tratamientos con APO-1 y MG132 favorecen la acumulación de p53 en el núcleo. **(A)** QE6-16 fueron teñidos para detección del núcleo (DAPI, fluorescencia azul) y p53 (tinción verde). QE6-16 fueron incubados con un anticuerpo anti-p53 toda la noche a 4 ºC. Las fotomicrografías fueron tomadas a las 6, 12 y 24 h después del tratamiento con 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos. **(B)** Cuantificación de la señal fluorescente de los experimentos de microscopia confocal. Cien células fueron examinadas y detectada la señal nuclear de p53 por cada muestra. La gráfica es el resultado de este análisis. Las gráficas representan la media ±DS de tres ensayos independientes (**p<0.05*). El análisis estadístico fue realizado entre cada grupo de tratamiento y el control (DMSO) o entre los diferentes grupos.

11.6. El restablecimiento de altos niveles de la proteína p53 en células QE6-16 tratadas con MG132 o APO-1 más MG132 induce la expresión de genes blanco de p53

Posterior a la inhibición del proteosoma 26S, en un corto tiempo (2 a 6 h) p53 induce la transcripción de sus genes blanco, incrementando los niveles de RNA mensajero y sus correspondientes proteínas involucradas en reparación de DNA, arresto del ciclo celular y muerte celular. Para determinar si la localización de p53 en el núcleo de QE6-16 después de los diferentes tratamientos se encuentra asociada con la inducción transcripcional de genes blanco de p53, se analizó la expresión de los genes que participan en apoptosis, como TP53INP1 (Tomasini y col., 2005), y Bax (Petre y col., 2007), ciclo celular (p21) (Petre y col., 2007) y autofagia (LC3). Como se muestra en la Figura 14, todos los genes mostraron un comportamiento similar después de los tratamientos. La expresión del RNA mensajero de p21 fue significativamente incrementado en células tratadas con MG132 o APO-1 más MG132 por 3 y 6 h; sin embargo, el más alto incremento en los niveles del RNA mensajero de p21 fue después de 6 horas de tratamiento con MG132 ($p<0.05$). Además, las células tratadas con APO-1 presentaron una disminución en la expresión del RNA mensajero del gen p21 a las 3 h y un ligero incremento a las 6 h. El RNA mensajero de TP53INP1 fue significativamente incrementado a las 3 y 6 h en células tratadas con MG132 o APO-1 más MG132 ($p<0.05$). Similares resultados para la expresión del RNA mensajero de Bax fueron observados a las 3 y 6 h en células tratadas con MG132 o APO-1 más MG132. Por otro lado, la expresión del RNA mensajero de LC3 presentó un considerable incremento a las 6 h en células tratadas con MG132 o APO-1 más MG132. Interesantemente, se observó un incremento significativo de los genes LC3B y p21 en el tratamiento con APO-1 a las 6 h ($p<0.05$). Estos resultados mostraron una inducción de genes blanco de p53, principalmente en los tratamientos en los cuales el restablecimiento de los niveles de p53 fueron detectados.

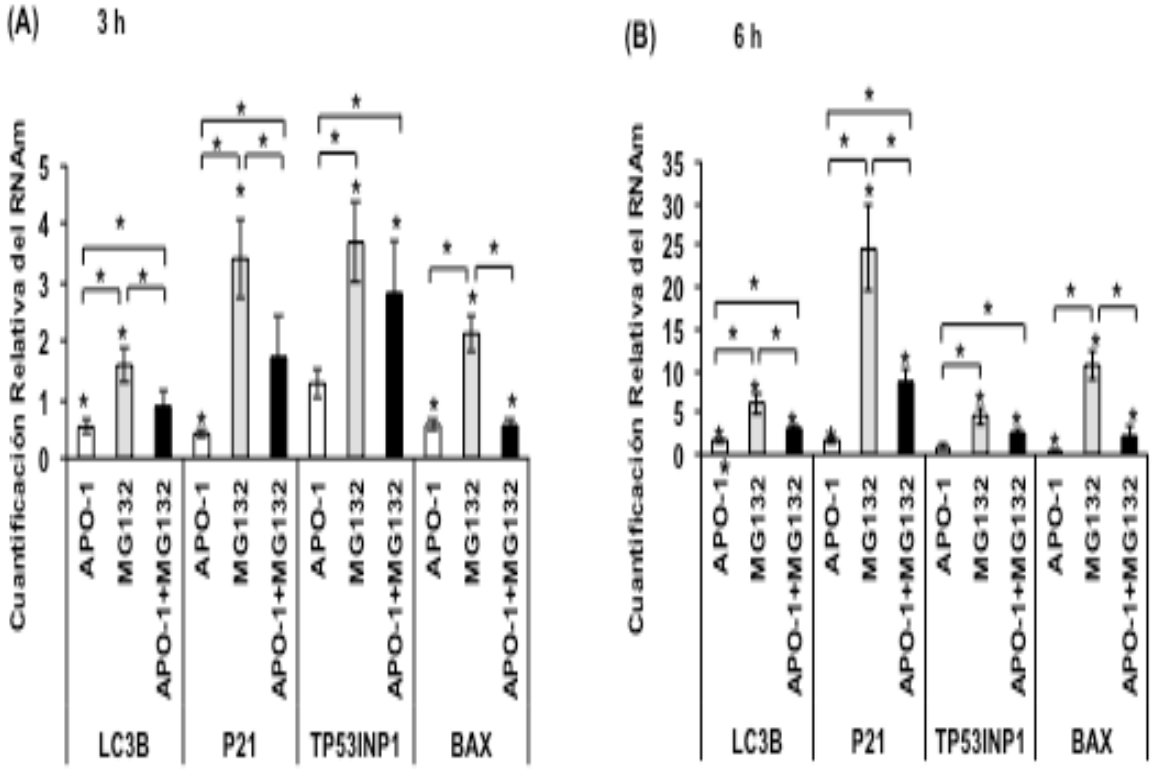


Figura 14. Expresión de genes blanco de p53 en QE6-16 incubados con MG132 y APO-1 determinados por RT-qPCR. QE6-16 incubados con 20 μM de MG132, 100 ng/ml APO-1 o ambos compuestos por **(A)** 3 h o **(B)** 6 h. Las células cultivadas en presencia de DMSO fueron utilizadas como calibrador para normalizar los genes evaluados. Los datos fueron analizados con la ecuación: cantidad de blanco= $2^{-\Delta\Delta CT}$ [22]. La media ±DS de tres experimentos biológicos independientes fueron realizados por duplicado. Las diferencias significativas encontradas entre los diferentes tratamientos y el grupo control (DMSO), o entre los diferentes grupos son indicados con un asterisco, $*p<0.05$.

11.7. La inducción de apoptosis por MG132 y APO-1 es dependiente de la actividad transcripcional de p53 y no requiere de la actividad transcripcional de c-Myc.

Para explorar el papel de la activación de p53 en la apoptosis inducida por APO-1 y MG132, a continuación se investigó el efecto de la pifitrina-α (PFT-α, un inhibidor de la actividad transcripcional de p53) (Murphy y col., 2004), sobre la actividad de caspasa-3 (como un indicador de la apoptosis). Los resultados observados sugieren que 2 h de preincubación de las células con 10 μM de PFT-α, significativamente disminuyen la actividad de caspasa-3 en células tratadas con APO-1, MG132, o APO-1 más MG132 comparadas con células que no fueron preincubadas con PFT-α (Figura 15A)(p<0.05). La reducción en la actividad de caspasa-3 por PFT-α fue más evidente en células tratadas con MG132 (3.7 veces) y APO-1 más MG132 (3 veces) comparada con células tratadas con APO-1 (2.8 veces). Este resultado muestra que p53 es un inductor de la apoptosis en células incubadas en presencia de MG132 y APO-1 más MG132, lo cual fue observado por la reducción en la actividad de caspasa-3 (un ejecutor primario de la apoptosis).

Como previamente fue mencionado, c-Myc es una proteína que es degradada por E6. Debido a que también se observó un incremento en los niveles de la proteína c-Myc en células QE6-16 incubadas en presencia de MG132 y APO-1 más MG132 en los diferentes tiempos de tratamiento (Figura 15B), en la presente tesis se evaluó el papel de c-Myc en la apoptosis inducida por APO-1 y MG132. Los resultados muestran una discreta pero significativa reducción en la apoptosis en la presencia del inhibidor de dimerización de Myc/Max (10058-F4) (Figura 15B). Este resultado sugiere un papel discreto en la apoptosis inducida por c-Myc en células tratadas con APO-1 más MG132 comparada con los resultados observados en p53.

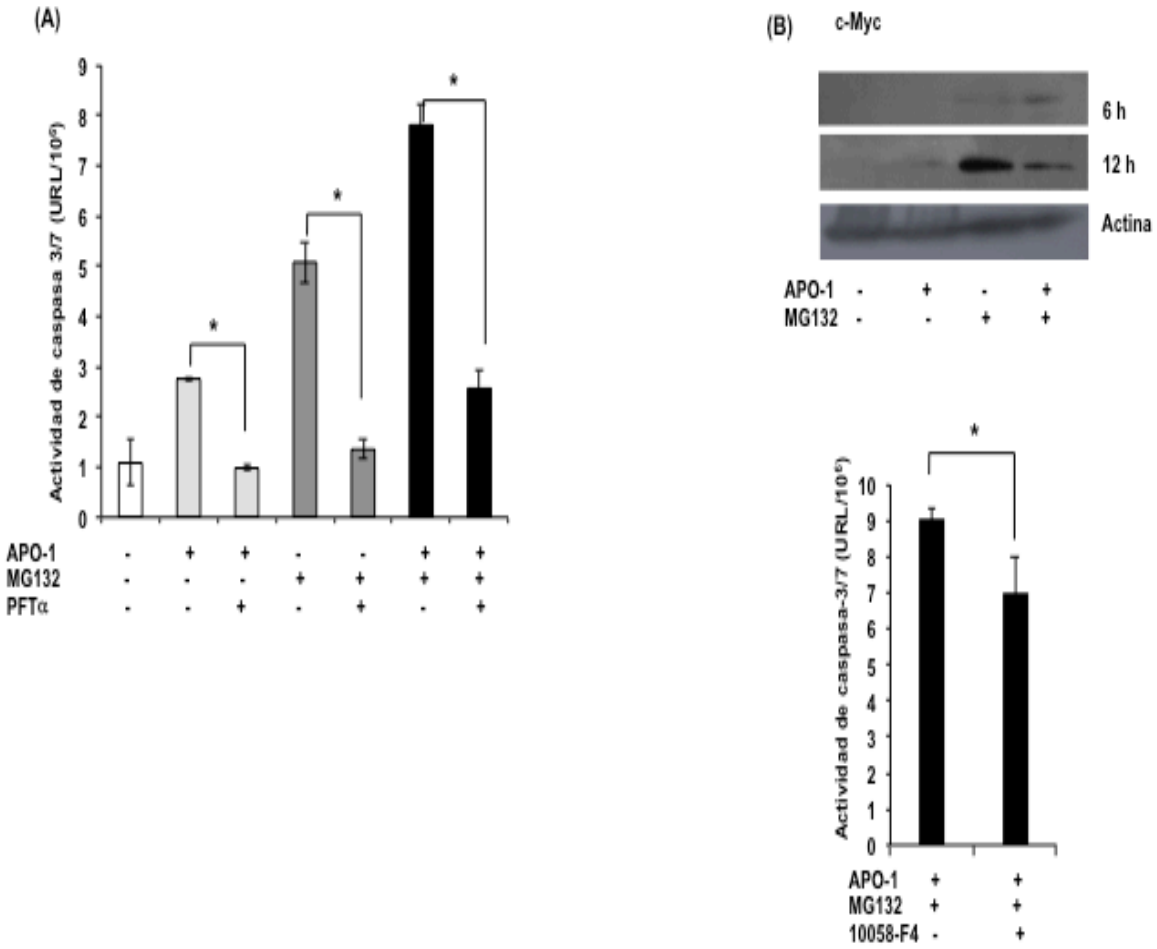


Figura 15. Efecto de pifitrina alfa (PFT-α) y 10058-F4 en la apoptosis inducida por MG132 y APO-1. **(A)** QE6-16 fueron cultivados en presencia de 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos durante 24 h, en presencia o ausencia de 10 μM de PFTα (un inhibidor de la actividad de p53). **(B)** Inmunodetección (Western blot) de la proteína c-Myc utilizando 50 μg de proteína total/carril de QE6-16 cultivados en presencia de 20 μM de MG132, 100 ng/ml de APO-1 o ambos compuestos durante 6 y 12 h. En las mismas condiciones de PFTα, QE6-16 fueron incubados en presencia de MG132 y APO-1 en ausencia o presencia de 10058-F4 (inhibidor de la dimerización Myc/Max). La actividad de caspasa-3/7 fue determinada por hidrólisis del substrato luminogénico que contiene la secuencia DEVD. Las lecturas fueron realizadas a las 0.5 h adicionando el reactivo de caspasa; la luminiscencia es proporcional a la actividad de la caspasa-3/7 expresada como URL. El valor del control (blanco que corresponde al pozo sin células) fue restado a cada muestra. Cada punto representa el promedio de los triplicados. Se indican las diferencias significativas encontradas entre APO-1 más MG132 vs. APO-1 más MG132 más 10058-F4, *$p<0.05$.

Finalmente, para determinar la especificidad del tratamiento, se realizaron ensayos de citotoxicidad en las líneas celulares derivadas de cáncer cervicouterino (HeLa y SiHa), cultivo primario de queratinocitos normales (QN) y células QE6-16. El tratamiento de las líneas celulares mencionadas previamente con 20 μM de MG132 y 100 ng/ml APO-1 más MG132 durante 24 h inhibió el crecimiento de células HeLa (35 % y 81 %), SiHa (45 % y 41 %), QE6-16 (49 % y 89 %) y QN (31 % y 67 %). El tratamiento solo con APO-1 presentó un efecto nulo o discreto sobre la proliferación de las líneas celulares (Figura 16).

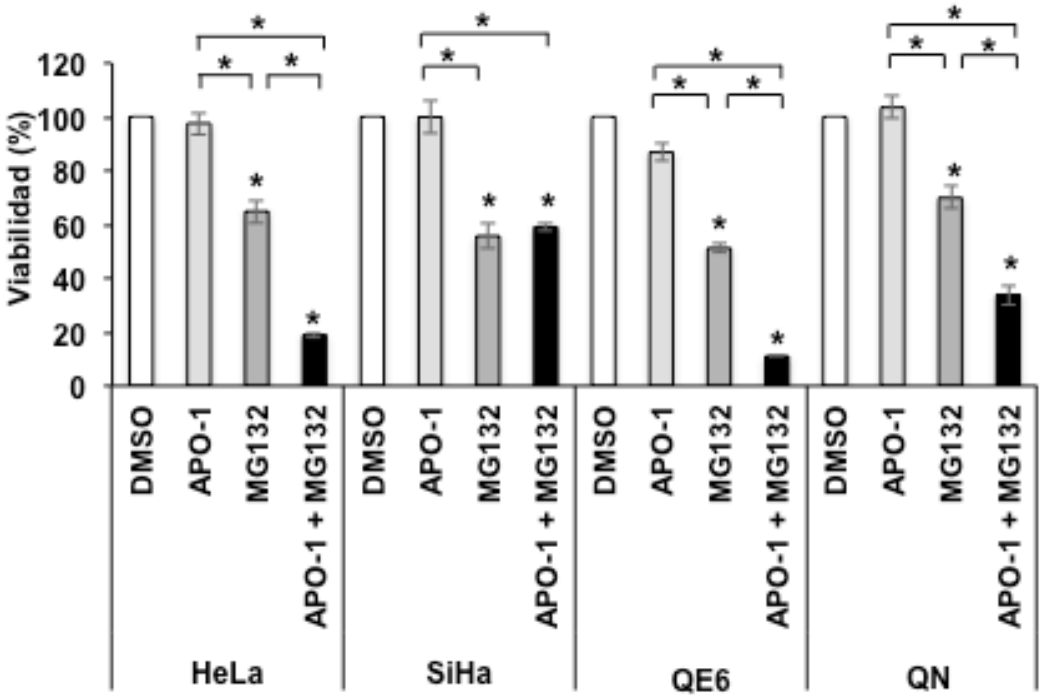


Figura 16. Efecto citotóxico de APO-1 más MG132 en líneas celulares derivadas de CaCU, queratinocitos normales y QE6-16. Todas las células fueron incubadas con 20 μM de MG132 (inhibidor del proteosoma), 100 ng/ml de APO-1, o ambos compuestos por 24 h. El número de células viables fue determinado a través de un ensayo de proliferación celular no-radioactivo (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, MTS). Las intensidades colorimétricas fueron medidas por absorbancia en Labsystems Multiskan MS. Las gráficas representan la media ±DS de tres ensayos independientes. El asterisco representa los resultados estadísticamente diferentes del control (DMSO) (*$p<0.05$).

12. DISCUSIÓN

En este estudio, se encontró que el restablecimiento de la expresión y actividad de la proteína p53 mediado por APO-1 y MG132 favorecen la inducción de apoptosis y autofagia en queratinocitos humanos inmortalizados con E6 (QE6-16). Posterior al tratamiento la proteína p53 se localizó en el núcleo, lo cual coincide con la inducción transcripcional de sus genes blanco que participan en arresto del ciclo celular, apoptosis y autofagia. Previamente se sugirió que el restablecimiento de la expresión de la proteína p53 sensibiliza a QE6-16 a apoptosis mediada por la activación de la vía de CD95, y que la inactivación del proteosoma 26S en un corto período de tiempo no induce apoptosis (Aguilar-Lemarroy y col., 2002). Sin embargo, nosotros encontramos que el tratamiento solamente con MG132 durante 24 h es suficiente para inducir apoptosis, la cual es dependiente de la actividad transcripcional de p53 (inhibida por PFT-α).

Por otro lado, se ha demostrado que las oncoproteínas E6-AR degradan diversas proteínas proapoptóticas (FADD, TNF-R1, procaspasa-8 Bax y Bak), inhibiendo la apoptosis (Lagunas-Martínez y col., 2010). En relación con la inactivación de las proteínas proapoptóticas por E6-AR, es posible que sea necesario bloquear primero la actividad del proteosoma 26S (por ejemplo con MG132) (Aguilar-Lemarroy y col., 2002; Hougardy y col., 2006) para restablecer los niveles de proteínas proapoptóticas, y posteriormente inducir la vía extrínseca de la apoptosis con CD95L o un anticuerpo que imite la acción CD95L o TNF-alfa. Debido a que p53 induce la apoptosis principalmente a través de la vía intrínseca, nuestros hallazgos sugieren que esta vía es la principalmente activada en la apoptosis inducida por APO-1 más MG132, posiblemente a través del fraccionamiento de Bid (tBid).

Consistente con los resultados obtenidos en esta tesis, se reportó que MG132 sensibilizó a las líneas celulares HeLa y SiHa a apoptosis inducida por TRAIL, desafortunadamente la autofagia o fosforilaciones en p53 no fueron analizadas en este estudio (Hougardy y col., 2006). Además, células de osteosarcoma humano cultivadas en presencia de TRAIL presentaron una baja tasa de apoptosis (Li y col., 2013). En contraste, MG132 o MG132 más TRAIL drásticamente incrementaron la apoptosis en células de osteosarcoma y glioma humano (Seol y col., 2011; Li y col., 2013). Estos resultados sugieren que en las vías de Fas o TRAIL, los inhibidores del proteosoma 26S son necesarios (además del ligando) para

inducir la vía extrínseca de la apoptosis. Con respecto a la vía intrínseca de la apoptosis, se encontró que el potencial de la membrana mitocondrial disminuyó con los tratamientos de MG132 y APO-1 más MG132 (Figura 10B). La disminución del potencial de membrana mitocondrial podría ser explicada por la activación de la vía de p53 a través de MG132, además de la estabilización y activación de otras proteínas celulares, las cuales son requeridas para activar la apoptosis. Sin embargo, los experimento con PFT-alfa y 10058-F4 (un inhibidor de la dimerización de c-Myc/Max) sugiere que p53 juega un papel importante en la inducción de la apoptosis intrínseca.

Se ha reportado que E6 se une a FADD y protege a las células de la apoptosis inducida por señalización de CD95 (Filippova y col., 2004), inhibiendo la activación de caspasa-8 y -3. Los resultados encontrados en este trabajo muestran que los tratamiento con APO-1, MG132 y APO-1 más MG132 inducen la activación de caspasa-8 y -9 en QE6-16 (Figura 9C y 9D), sugiriendo que aun en la presencia de E6, todos los tratamientos inducen ambas vías de la apoptosis (intrínseca y extrínseca) en diferentes proporciones.

Es posible que la activación de la vía intrínseca por p53, en QE6-16 cultivados en presencia de APO-1 y MG132 una ligera activación de capasa-8 debería favorecer la fragmentación de Bid para generar la forma truncada activa (tBid) y colaborar en promover la activación de la vía intrínseca de la apoptosis (Kroemer y Reed, 2000). Los resultados obtenidos en esta tesis muestran una disminución en los niveles de la proteína Bid completa (22 kDa), principalmente en células tratadas con APO-1 más MG132. Estos resultados sugieren que Bid transmite una señal apoptótica del receptor CD95 a la mitocondria fortaleciendo la activación de la vía intrínseca.

En CaCU pocos reportes han sugerido inhibición de autofagia mediada por HPV (Salazar-León y col., 2011; Hanning y col., 2013), pero el mecanismo a través del cual las oncoproteínas virales inhiben este tipo de muerte es desconocido. Interesantemente, en QE6-16 se observó inducción de autofagia en los tratamientos con MG132 y APO-1 más MG132 (Figura 11), lo cual es consistente con niveles incrementados de la proteína p53. A la fecha se desconoce si el sólo tratamiento con APO-1 induce autofagia en otras líneas epiteliales; sin embargo, los resultados de este trabajo muestran una cooperación en células QE6-16 tratadas con APO-1 más MG132 para inducir la autofagia. Además, una fuerte lipidación de LC3B fue observada a través de Western blot, confirmando que los

tratamientos con MG132 y APO-1 más MG132 en QE6-16 favorecen la activación de la autofagia.

La autofagia puede ser parte de la cascada de eventos que guía a la muerte celular, ya sea colaborando con otros mecanismos de muerte celular o causando muerte celular por si misma (Nikoletopoulou y col., 2013). En QE6-16 tratados con APO-1 más MG132 se observó una inducción de la apoptosis 3 veces mayor que la observada en autofagia (Figuras 9B y 11B). Este resultado podría sugerir la importancia de la apoptosis en la muerte celular mediada por APO-1 más MG132 comparada con la menor contribución de la autofagia.

Los resultados obtenidos demuestran que p53 nuclear tiene un papel como factor de transcripción proapoptótico y proautofágico. Adicionalmente, se ha reportado que p53 citoplasmático suprime la autofagia en diversas condiciones experimentales, y que la inactivación de p53 puede inducir autofagia (Tasdemir y col., 2008). Estos resultados demuestran una directa relación entre la localización celular de p53 y activación/inhibición de autofagia. Así, los resultados obtenidos sugieren que p53 nuclear induce muerte celular principalmente mediada por apoptosis con una menos contribución de la autofagia.

p53 es una proteína de vida corta, con bajos niveles en células de mamífero sin estrés (Jenkins y col., 2012). Posterior a un evento de estrés celular, p53 se estabiliza y se activa a través de diferentes modificaciones postraduccionales, tales como las fosforilaciones en Ser15 y Ser20 (Meek y Anderson., y 2009). La fosforilación en Ser46 es quizá el ejemplo más claro de una modificación postraduccional en p53 que es crítica para la inducción de sus genes blanco proapoptóticos. De manera interesante, QE6-16 cultivados en presencia de APO-1, MG132 y APO-1 más MG132 mostraron una débil fosforilación de p53 en Ser20 y niveles incrementados de fosforilación en Ser46, sugiriendo que la inducción de la apoptosis intrínseca y/o extrínseca podría requerir altos niveles de p53 y fosforilaciones en varios residuos de serina de p53.

La fosforilación de p53 en Ser46 correlaciona con los resultados observados en la Figura 14, en la cual se observó que la reactivación de p53 retiene la habilidad para inducir genes blanco proapoptóticos, tales como p21 y Bax (Petre y col., 2007), y TP53INP1 (Tomasini y col., 2005). Similar a los resultados encontrados, Gareau y col., reportó que el RNA

mensajero de p21 es inducido en células HeLa tratadas con Bortezomib (inhibidor del proteosoma) durante periodos de 4 y 10 h (Gareau y col., 2011).

Similar a lo observado con el RNA mensajero de p21, el RNA mensajero de TP53INP1 incremento a las 3 y 6 h en QE6-16 cultivados en presencia de MG132 o APO-1 más MG132. Debido a que TP53INP1 promueve la autofagia (N´guessan y col., 2011; Seillier y col., 2012), es posible que el incremento del RNA mensajero a las 3 y 6 h también favorezca la autofagia (Seillier y col., 2012). Además, ha sido reportado que TP53INP1 fosforila a p53 en Ser46 favoreciendo la estabilidad y promoviendo la unión de p53 a los promotores de genes proapoptóticos más que a los genes que participan en reparación del ADN (D'Orazi y col., 2012).

Ortiz-Lazareno y colaboradores encontraron que el RNA mensajero de Bax incrementó en células U937 tratadas con MG132 y MG132 más Doxorrubicina comparado con el grupo de células no tratadas (Ortiz-Lazareno y col., 2014), dichos resultados son similares a los encontrados en esta tesis. Los resultados de lipidación de LC3B y el incremento del RNA mensajero de LC3B principalmente en QE6-16 tratados con MG132 y APO-1 más MG132 demuestran la activación de la autofagia.

Resultados observados en la inducción de apoptosis en células QE6-16 por el tratamiento individual o combinado sugieren que la apoptosis es principalmente dependiente de la actividad transcripcional de p53 debido a que 10 μM de PFT-alfa suprime este proceso (Figura 15A); similares resultados fueron observados utilizando 30 μM de PFT-alfa (Dato no mostrado). Una disminución del 78 % en la actividad de capasa-3/7 fue observada cuando los QE6 fueron tratados con PFT-α (Figura 15A). Estos resultados demuestran claramente la importancia de la actividad transcripcional de p53 en la vía de la apoptosis intrínseca. Sin embargo, el restante 22 % de la actividad de estas caspasas podría ser generado por la activación de vías independientes de p53. Por esta razón, se evaluó el posible papel de c-Myc en la apoptosis inducida por APO-1 más MG132, encontrando que c-Myc tiene un papel discreto en inducir la apoptosis por estos compuestos.

13. CONCLUSION

CaCU es un problema de salud pública a nivel mundial. Actualmente, la FDA ha aprobado 3 vacunas (Gardasil, Gardil 9 y Cervarix) para prevenir la infección por el VPH. Sin embargo, los efectos en la disminución de la mortalidad del CaCU podrían tardar años. Por lo que nuevas estrategias terapéuticas que incluyan dos o más compuestos citotóxicos deben de ser analizados.

En conclusión, se demostró que APO-1 y MG132 cooperan para restablecer la actividad de p53 e inducir la apoptosis dependiente de p53 y la autofagia en células QE6. El uso combinado de inhibidores del proteosoma en bajas concentraciones con diversos compuestos terapéuticos podría tener un futuro alentador en el tratamiento del CaCU.

14. PERSPECTIVAS

Incorporar MG132 y compuestos terapéuticos en nanoparticulas para liberarlos específicamente en células que expresan a la oncoporteína E6 de alto riesgo.

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16. ARTÍCULOS

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MG132 plus apoptosis antigen-1 (APO-1) antibody cooperate to restore p53 activity inducing autophagy and p53-dependent apoptosis in HPV16 E6-expressing keratinocytes

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Abstract The E6 oncoprotein can interfere with the ability of infected cells to undergo programmed cell death through the proteolytic degradation of proapoptotic proteins such as p53, employing the proteasome pathway. Therefore, inactivation of the proteasome through MG132 should restore the activity of several proapoptotic proteins. We investigated whether in HPV16 E6-expressing keratinocytes (KE6 cells), the restoration of p53 levels mediated by MG132 and/or activation of the CD95 pathway through apoptosis antigen-1 (APO-1) antibody are responsible for the induction of apoptosis. We found that KE6 cells underwent apoptosis mainly after incubation for 24 h with MG132 alone or APO-1 plus MG132. Both treatments activated the extrinsic and intrinsic apoptosis pathways. Autophagy was also activated, principally by APO-1 plus MG132. Inhibition of E6-mediated p53 proteasomal degradation by MG132 resulted in the elevation of p53 protein levels and its phosphorylation in Ser46 and Ser20; the p53 protein was localized mainly at nucleus after treatment with MG132 or APO-1 plus MG132. In addition, induction of its transcriptional target genes such as p21, Bax and TP53INP was observed 3 and 6 h after treatment. Also, LC3 mRNA was induced after 3 and 6 h, which correlates with lipidation of LC3B protein and induction of autophagy. Finally, using pifithrin alpha we observed a decrease in apoptosis induced by MG132, and by APO-1 plus MG132, suggesting that restoration of APO-1 sensitivity occurs in part through an increase in both the levels and the activity of p53. The use of small molecules to inhibit the proteasome pathway might permit the activation of cell death, providing new opportunities for CC treatment.

Keywords HPV · Apoptosis · Autophagy · APO-1 · HPV16 E6 · Phospho-p53 Ser46

Vicente Madrid-Marina and Patricio Gariglio have contributed equally to this work.

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Introduction

The High Risk Human papillomaviruses (HR-HPVs) have been identified as the major cause of Cervical Cancer (CC) [1]. HPV16 is commonly associated with lesions that can progress to carcinoma [2]. Upon infection, viral oncoproteins (E6 and E7) can interfere with several processes such as transcription regulation, immune response, cellular adhesion, proliferation and apoptosis [3]. The best-described target for E6 is the p53 tumor suppressor protein; the interaction between E6 and p53 promotes the degradation of this cellular protein through an ubiquitin-dependent mechanism [4]. However, additional mechanisms of apoptosis inhibition

by HR-E6 involve the proteolytic inactivation through ubiquitination of different proapoptotic proteins such as c-Myc [3], Bak [5], FADD [6] and procaspase-8 [7].

Apoptosis is a form of cell death that is regulated physiologically and genetically [8] and contributes to the elimination of chemotherapy damaged cells and those infected with virus and intracellular parasites [9–11]. Abnormal apoptosis is involved in various diseases such as autoimmune diseases [12] and cancer [13]. Two main apoptotic routes have been identified [8]: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. For major details about apoptosis pathways check a review published by Galluzzi et al. [14].

It is known that apoptosis-independent cell death pathways can be activated following cell damage. One of these pathways is known as autophagic or lysosomal cell death (type II cell death). The high content of hydrolytic enzymes in lysosomes makes them potentially harmful to the cell. During autophagy, specific genes that induce this type of cell death are expressed and cytoplasmic constituents (including organelles) are delivered through both macro- and microautophagy to lysosomes to promote their degradation [15]. A classical hallmark of autophagy is the posttranslational modification of LC3B by lipidation, which allows its association with autophagic vesicles [16]. On the other hand, the activation of cell death might reflect a crosstalk between the processes of autophagy and apoptosis. Thus, although autophagy and apoptosis clearly represent distinct cellular processes with fundamentally different biochemical and morphological features, the protein networks that control their regulation and execution can be highly interconnected [17, 18].

In a previous study, it was reported that E6 immortalized keratinocytes (KE6 cells) were resistant to CD95 ligand while E7 immortalized keratinocytes were sensitive [19]. The KE6 cells were sensitized to ligand-induced cell death by inhibition of the 26S proteasome complex through MG132. Coincidentally, a subsequent re-expression of p53 and c-Myc proteins was observed after treatment with MG132 [19]. Due to the inhibition of apoptosis by E6 through p53 degradation, we work only with cells expressing the viral E6 oncogene. Therefore, the main aim of this study was to determine whether p53 plays a decisive role in the induction of cell death mediated by APO-1 after inhibition of the 26S proteasome complex in E6-expressing keratinocytes. In the present report, employing KE6 cells we found that p53 is necessary in MG132 and APO-1-induced apoptosis. We localized the p53 protein at the nucleus following different treatments, and we demonstrated that some p53 targets are transcriptionally activated, suggesting again p53 dependent apoptosis. In addition, we evaluated in KE6 cells the role of c-Myc in MG132 and APO-1-induced apoptosis, finding under these conditions only a slight decrease in apoptosis after inhibition of c-Myc transcriptional activity, suggesting a discrete role of c-Myc in the apoptosis induced by these compounds.

Materials and methods

Reagents

Keratinocyte-Serum Free Medium (K-SFM) and supplements (EGF and bovine pituitary extract) were purchased from Gibco (Grand Island, NY, USA). The ClearMount mounting solution was obtained from Invitrogen (Frederick, MD, USA) and CAS-Block was obtained from Zymed (San Francisco, CA, USA). The inhibitor specific to p53 transcriptional activity (PFT-α), 4′,6-diamidino-2-phenylindole (DAPI), as well as Acridine Orange (AO) and tetramethylrhodamine ethyl ester (TMRE) were purchased from Sigma Aldrich (St Louis, MO, USA). The proteasome inhibitor MG132 was obtained from EMD Millipore (Billerica, MA USA), which was dissolved in DMSO and stored at −20 °C until use. The reagent that specifically inhibits the c-Myc-Max interaction, 10058-F4 was obtained from Calbiochem (Darmstadt, Germany). Annexin V-FITC Apoptosis Detection Kit was purchased from BioVision (Mountain View, CA, USA). The antibody that induces apoptosis, Anti-Fas (APO-1, clone CH11) was obtained from Merck-Millipore (Billerica, MA, USA). Antibodies against p53-HRP (Western Blot) and p53 (Confocal microscopy), and Phosphatase inhibitors were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Invitrogen (Camarillo, Ca, USA), respectively. The antibodies against phospho-p53 Ser46, phospho-p53 Ser20, Bid, c-Myc and LC3B were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin-HRP was purchased from Sigma (St Louis, MO, USA). The antibody Alexa Fluor 488 anti-rabbit was obtained from Life Technologies (Carlsbad, CA, USA).

KE6 cell culture

Human Keratinocytes immortalized with HPV16 E6 (KE6 cells) were proportioned by Dr. Frank Rösl (Heidelberg, Germany) and were previously reported [19]. KE6 cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in K-SFM supplemented with 20 µg/ml of Bovine Pituitary Extract, 0.1 ng/ml of Recombinant Epidermal Growth factor (Gibco®), 2% Fetal Calf Serum and an antibiotic–antimycotic mixture (Invitrogen™). For the different experiments, KE6 cells were incubated in the presence of DMSO (control), MG132 (20 µM), APO-1 (100 ng/ml) or both treatments (MG132 plus APO-1) for the indicated time.

Immunofluorescence staining

KE6 cells (5×10^4 cells per well) were grown on Lab-Tek 8-well plates (Nalge Nunc International, Roskilde, Denmark). After treatment, cells were washed with sterile PBS and fixed by incubation with 2% paraformaldehyde in PBS for 10 min at 4 °C, washed again with PBS and blocked with CAS-Block for 15 min at 37 °C. After removing CAS-Block, the well was washed with PBS. KE6 cells were incubated overnight at 4 °C with mouse anti-p53 antibody and then washed with PBS. After rinsing, KE6 cells were incubated in the dark for 2 h at 37 °C with an Alexa Fluor® 488 goat anti-rabbit IgG for the detection of primary antibody. In order to stain the nuclei, KE6 cells were incubated for 15 min with 100 ng/ml of DAPI in PBS. KE6 cells were washed with PBS and prepared with permanent mounting medium. Finally, the cells were analyzed using a confocal microscope Leica DMI 4000B with a 63× oil-immersion objective. For detection from Alexa® 488/DAPI we used the green–blue excitation laser. Files were analyzed using LAS AF Lite program. One hundred cells were examined to detect nuclear p53 signal for each treatment in the indicated time.

Apoptosis analysis

KE6 cells were grown in 6-well tissue culture plates (8×10^5 cells/well) and incubated for 24 h in the presence of the different treatments. KE6 cells were harvested, washed with PBS, and collected by centrifugation. Next, KE6 cells were resuspended in 100 µl of binding buffer and treated with Annexin V-FITC (5 µl) and 50 µg/ml propidium iodide (PI) (BioVision). KE6 cells were incubated at room temperature for 10 min in the dark, and 10,000 cells were processed in "FACSCaliburTM (BD Biosciences)". KE6 cells positive for Annexin V were detected using FITC signal detector (FL1) and PI positive cells were detected through the phycoerythrin emission signal detector (FL2). Analysis was done on 10,000 events using the Summit V4.3 software.

Mitochondrial membrane potential detection assay

KE6 cells were seeded and treated as in the apoptosis assay. After treatment for 24 h, KE6 cells were detached and incubated in the presence of 200 nM TMRE (Tetramethylrhodamine Ethyl Ester) for 15 min at 37 °C. KE6 cells were washed with PBS and the fluorescence intensity was analyzed through "FACSCaliburTM (BD Bioscience)" flow cytometer using the FL2 signal detector. Analysis was performed on 10,000 events using the Summit V4.3 software.

Lysosome integrity assessment

KE6 cells were seeded and treated for the flow cytometer assays as described above. For this assay, KE6 cells were detached and incubated with 5 µg/ml of AO (Acridine Orange) for 15 min at 37 °C. KE6 cells were washed with PBS and 10,000 cells were analyzed through "FACSCaliburTM (BD Bioscience)" flow cytometer using the FL3 signal detector. Analysis was carried out using the Summit V4.3 software.

Caspases activity assay

The Caspase-Glo assay kit (Promega, Madison, WI) was used to measure the executioner caspases-3/7, and initiator caspases-8, -9 activities. Each well of a 96 well/culture plate contained 10,000 KE6 cells in K-SFM; the plate was stirred at 300 rpm for 30 s and then incubated at room temperature with 100 µl Caspase-Glo reagent for 30 min. A blank reaction was included which only contained cell culture medium without cells. The luminescence of each sample was measured in a plate-reading luminometer (Glomax, Promega, Madison WI).

Western blot analysis

After treatments, KE6 cells (8×10^5 cells/well) were washed and lysed with RIPA buffer supplemented with both protease and phosphatase inhibitors, and incubated for 20 min on ice. Protein extracts were obtained by centrifugation at 12,000 rpm for 20 min. The protein concentration in cleared lysates was measured using the BCA Protein Assay kit (Thermo Scientific, Rockford, IL) and 50 µg total proteins were separated through 10 and 15% SDS polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked 1 h with 5% nonfat milk and incubated with anti-p53-HRP overnight at 4 °C. To detect Bid, c-Myc, LC3B, p53 Ser20 and p53 Ser46 we used an anti-rabbit-HRP secondary antibody. Then, the membranes were washed three times with 0.1% Tween-20/PBS. P53 and actin were visualized using SuperSignal West Pico chemiluminiscence (Pierce, Thermo Scientific, Rockford, Illinois, USA), and Bid, c-Myc, LC3B, p53 Ser20 and p53 Ser46 were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific, Rockford, Illinois, USA) and recorded on X-ray film (Kodak, St. Louis MO, USA). Anti-actin-HRP was used as loading control. We used ImageJ software for the densitometric analysis of p53 protein.

RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and its integrity was determined by electrophoresis in 1 % agarose gels. RNA concentration and purity (260/280) was evaluated using NanoDrop LITE Spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized using 1 µg of total RNA and 200 U M-MLV reverse transcriptase (Invitrogen) in the presence of oligo-dT primer (Invitrogen) in standard conditions. Primers specific to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene were designed (Primer Express V3.0 software, Applied Biosystems) and used to verify synthesized cDNA integrity. The expression of E6 mRNA in KE6 cells was analyzed as previously reported [19] (Online Resource 1). PCR reactions were carried out in a final volume of 25 µl containing 1 µl of cDNA, 2.5 mM of dNTP Mix, 1 U of recombinant Taq DNA polymerase, 1× PCR Buffer, 2.5 mM MgCl2, and 10 pMol of each primer. All reagents were obtained from Invitrogen.

Quantitative real-time PCR

cDNA was obtained as in the RT-PCR section and diluted 1:10. Quantitative real-time PCR was performed with 2 µl of each diluted cDNA product. The reaction was done using SYBR Green PCR Master Mix (Applied Biosystems) according to the protocol provided by the manufacturer. Amplification of p53 gene targets was carried out with the primers shown in Table 1.

Hypoxanthine phosphoribosyl transferase (HPRT1) was obtained from Qiagen and was used to normalize the amount of p21, TP53INP1, Bax and LC3B mRNA present in each sample. The PCR program was as follows: 10 min at 95 °C; 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The specificity of the amplification products and the absence of primer dimers were determined by performing melting curve analyses in all cases. The standard curve for each gene was generated by five-fold serial dilutions cDNA obtained from KE6 cells treated with 20 µM MG132. The efficiency of PCR amplification for each gene was calculated using the standard curve method, $E = 10^{(-1/\text{slope})} - 1$. Relative expression was calculated using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) [20].

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical evaluation of significant differences was performed using the Wilcoxon-Mann-Whitney test. Differences of $p < 0.05$ were considered statistically significant. All analyses were performed using STATA version 12 (StataCorp, Collage Station, TX, EUA).

Results

MG132 and APO-1 induced both pathways of apoptosis (intrinsic and extrinsic) in E6-oncoprotein expressing human keratinocytes

We used E6-immortalized keratinocytes (KE6 cells) obtained and grown as indicated in "Materials and methods". We confirmed the E6 oncogene expression by RT-PCR assay. As shown in Online Resource 1, using E6-specific primers we observed the amplification of two bands of 344 and 161 bp in size, corresponding to the complete and the spliced viral mRNAs encoding E6 and E6*, respectively [19]. Next, we incubated KE6 cells in the presence of 20 µM MG132 (a proteasome inhibitor), 100 ng/ml APO-1 (an activator of the apoptotic response), or both, for 6, 12 and 24 h and apoptosis was determinated by flow cytometry using the Annexin V/PI assay. We did not find a significant induction of apoptosis at 6 and 12 h (data not shown) but clear induction was observed at 24 h. Therefore, all our incubations to determinate cell death were performed at 24 h. We observed the higher increase in the induction of apoptosis (47 %) after inactivation of the proteasome by MG132 and activation

Table 1 Sequence of primers used for real time PCR

PCR type	Primer	Sequence (5′→3′)	References
Real time	TP53INP1-F	GCACCCTTCAGTCTTTTCCTGTT	[23]
	TP53INP1-R	GGAGAAAGCAGGAATCACTTGTATC	
	Bax-F	GGGGACGAACTGGACAGTAA	[24]
	Bax-R	CAGTTGAAGTTGCCGTCAGA	
	p21-F	GGAAGACCATGTGGA CCTGT	[24]
	p21-R	GGC GTT TGG AGT GGT AGA AA	
	LC3B-F	AGGGTAAACGGGCTGTGTGA	
	LC3B-R	CCCCTGCAAGAGTGAGGACTT	Primer express V3.0 software

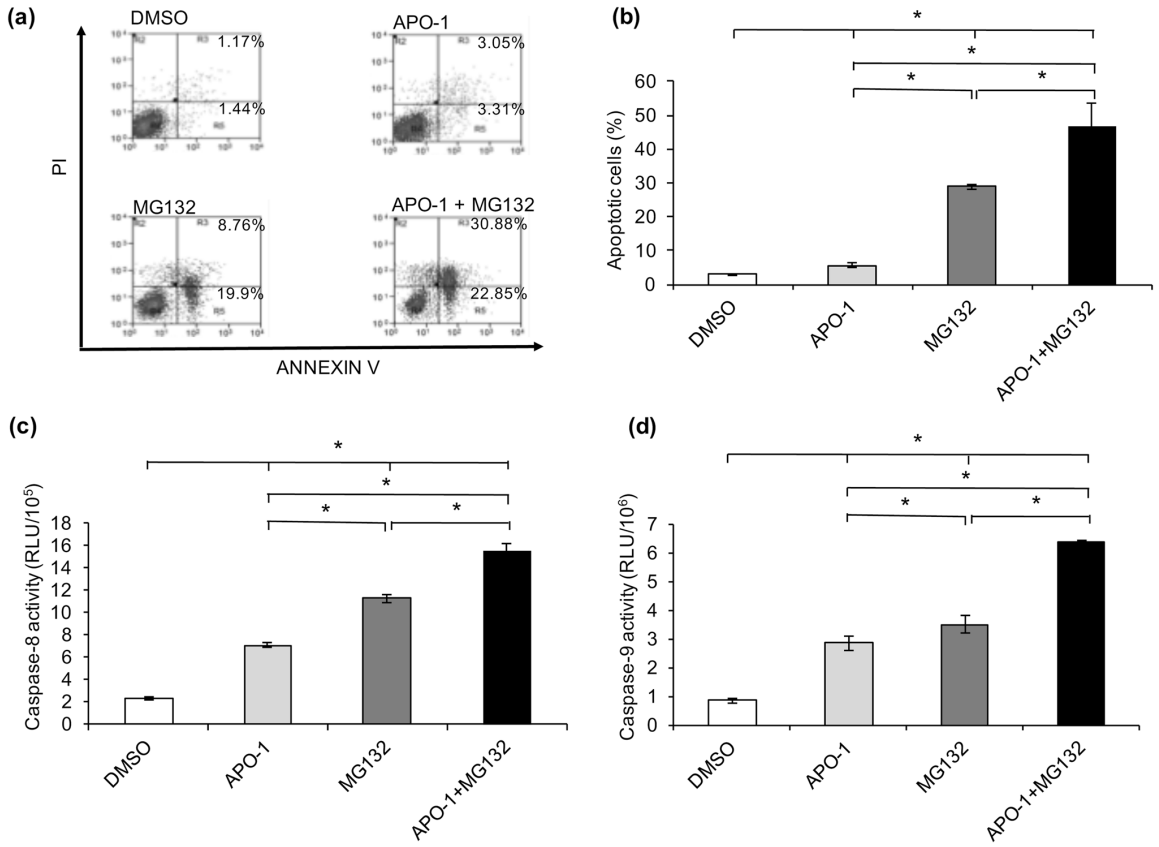

Fig. 1 Induction of apoptosis in KE6 cells incubated 24 h with APO-1 and MG132. Cells were incubated with 20 µM MG132 proteasome inhibitor, 100 ng/ml APO-1 (an antibody that mimics the CD95 ligand and induces the apoptotic response), or both, for 24 h and then stained with a combination of Annexin V and propidium iodide (PI) to detect apoptotic cells. **a** Representative flow-cytometry histograms of apoptosis using Annexin V. **b** Quantification of fluorescence intensities were measured by flow cytometry using FL1 (Annexin V) and FL2 (PI) channels as described in "Materials and methods". Caspase-8 (**c**) and caspase-9 (**d**) activity was determined by hydrolysis of the luminogenic substrate containing the DEVD sequence as indicated under "Materials and methods" readings were taken 0.5 h after adding the caspase substrate. Under these conditions, luminescence is proportional to the caspase-8 or -9 activity expressed as relative light units (RLU). The value obtained from the control without cells was subtracted from each RLU determination. The graphics represent the mean ± SD from three independent assays (*$p < 0.05$). Statistical analyses were carried out between each treatment with the control (DMSO) or among the different groups

of the extrinsic pathway of apoptosis mediated by APO-1 (MG132 plus APO-1) (Fig. 1a, b). However, treatment with MG132 alone also shows an elevated induction of apoptosis of about 29%, while APO-1 treatment just slightly increased apoptosis by a 6%. As expected, a low level of apoptosis was observed in the control group (DMSO).

To characterize the pathways of apoptosis activated by MG132 and APO-1, we analyzed the activation of the intrinsic and extrinsic pathways through caspase-9 or caspase-8 activation, respectively. After exposure to 100 ng/ml APO-1, 20 µM MG132 or a combination of 20 µM MG132 plus 100 ng/ml APO-1 for 24 h, we found that both pathways (Fig. 1c, d) were activated by the individual compounds (MG132 or APO-1) or when both molecules are combined (MG132 plus APO-1) ($p < 0.05$). However, in the combined treatment, the activation of caspase-8 and caspase-9 was much higher than in the individual groups or in the control group. We observed (Fig. 1d), the activation of the intrinsic pathway of apoptosis with all three treatments. Although the E6 oncoprotein degrades proteins involved in the activation of the death receptors, we found also activation of the extrinsic pathway in KE6 cells treated only with APO-1 (Fig. 1c). This result suggests that in the extrinsic pathway, apoptosis is initially activated through caspase-8, which should cleave full length Bid, resulting in activation of caspase-9 and the intrinsic pathway.

APO-1 plus MG132 favors activation of the intrinsic mitochondrial pathway in E6-oncoprotein expressing human keratinocytes

It is widely accepted that perturbations in the mitochondrial membrane contribute to apoptosis due to Cyt-c release. To confirm the participation of mitochondria in the apoptosis induction mediated by MG132 and APO-1 we analyzed the loss of mitochondrial membrane potential (ΔΨm) through flow cytometry using the cationic dye TMRE. We observed an important reduction in the membrane potential with the combined APO-1 plus MG132 treatment suggesting the Cyt-c release and apoptosis induction. Also, treatment with MG132 shows a high percentage in the reduction of the membrane potential and only a modest but significant change was observed after APO-1 treatment (Fig. 2a, b). Similar to apoptosis, TMRE assays showed an additive effect using APO-1 plus MG132 ($p < 0.05$). To identify the possible cleavage of Bid generating truncated tBid (a proapoptotic protein that favors the release of Cyt-c, and activation of the intrinsic apoptotic pathway during Fas signaling) we evaluated fragmentation of this protein through western blot. We observed a decrease in the full-length (22 kDa) protein levels at 12 and 24 h only in KE6 cells treated with APO-1 plus MG132 (Fig. 2c), suggesting that APO-1 participation in the induction of apoptosis in combination with MG132 is through the intrinsic pathway via Bid activation.

MG132 and APO-1 induced autophagy in E6-oncoprotein expressing human keratinocytes

In this assay, we analyzed the induction of autophagy by evaluating the permeability of lysosomal membranes through flow cytometry in the presence of Acridine Orange (AO) in KE6 cells treated with MG132 and APO-1. We found in the AO assay, a statistically significant increase in lysosomal permeability in KE6 cells treated with either

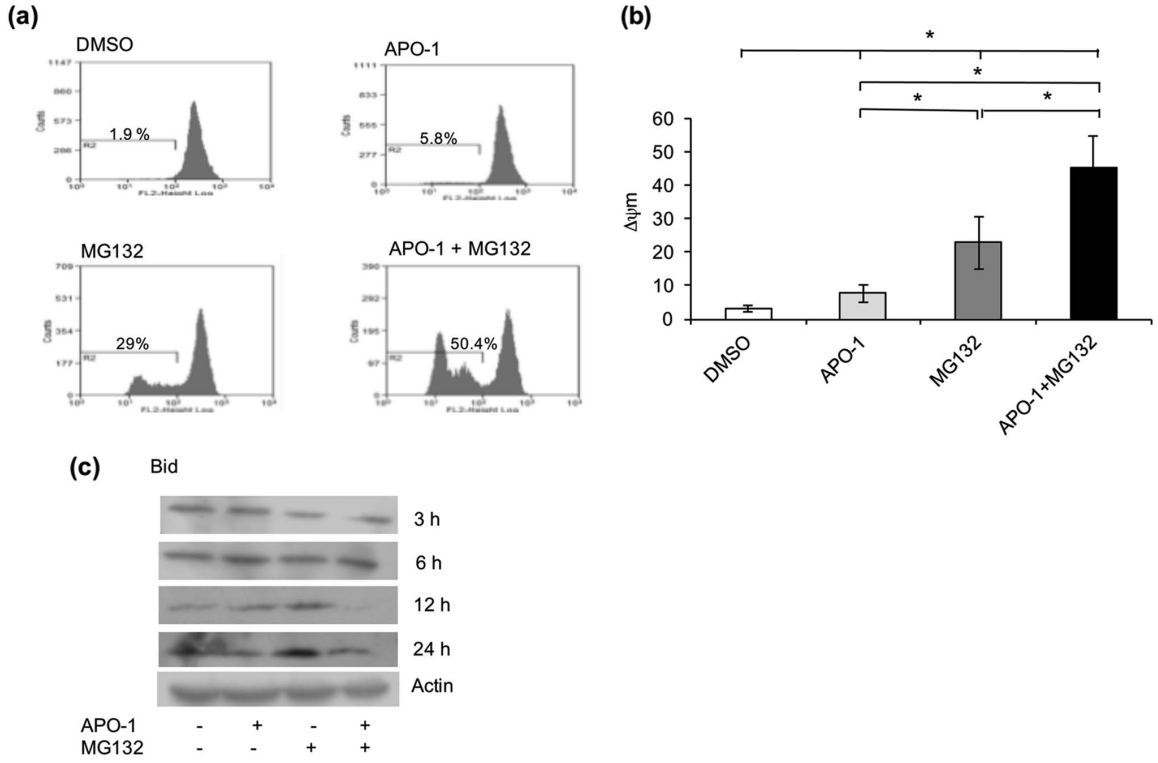

Fig. 2 Effect of APO-1 and MG132 on the mitochondrial membrane potential (Δψm) of KE6 cells. **a** Representative histograms for KE6 treated 24 h with 20 µM MG132, 100 ng/ml APO-1, or both. **b** Flow cytometry analysis of KE6 cells was similar to Fig. 1a. After 24 h cells were stained with TMRE fluorescent dye. The graphics represent the mean ± SD from three independent assays. *Asterisks* represent results statistically different from the control (DMSO) (*$p < 0.05$) or among the different groups. **c** Western blotting for Bid protein using lysates (50 µg protein/lane) from KE6 cells treated for 3, 6, 12 and 24 h with 20 µM MG132, 100 ng/ml APO-1, or both. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. The electrophoresis was performed in 15% SDS-PAGE gels as described under "Materials and methods". Results are representative of three independent experiments

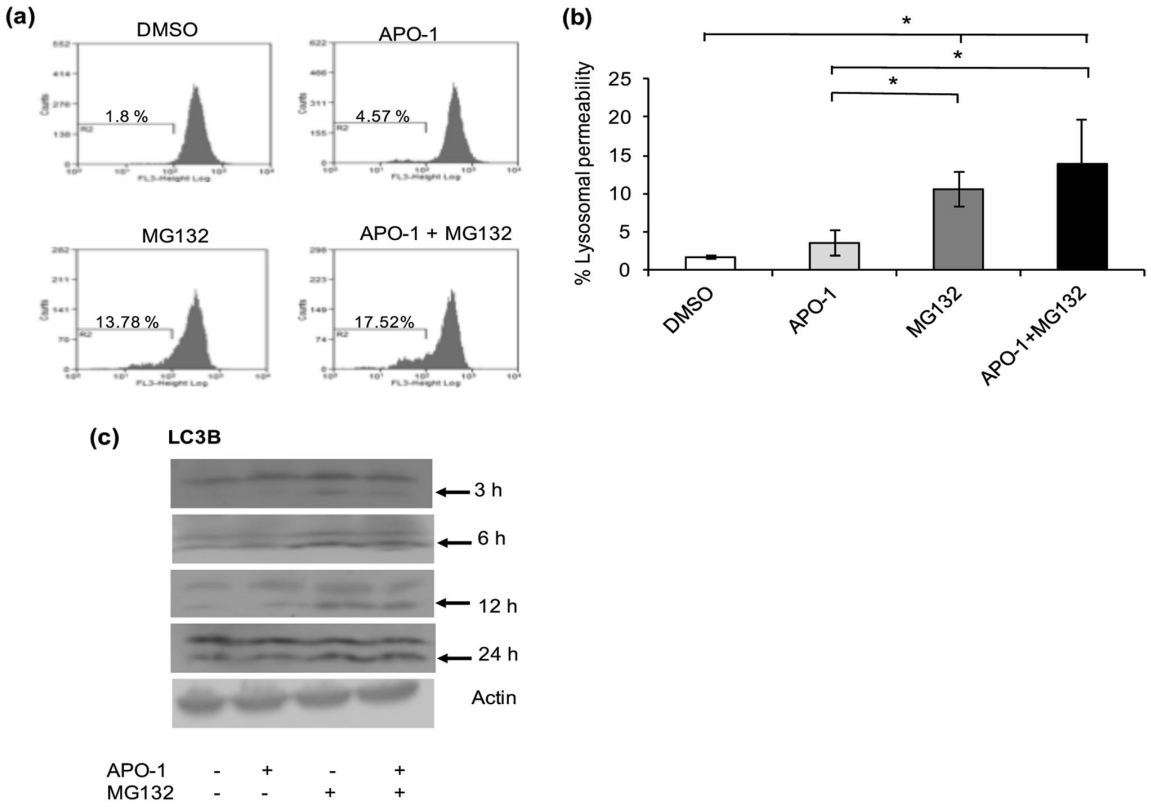

Fig. 3 Effect of MG132 and APO-1 on the lysosomal integrity and LC3B lipidation of KE6 cells. **a** Representative histograms for KE6 treated 24 h with 20 µM MG132, 100 ng/ml APO-1, or both. **b** Flow cytometry analysis was performed on KE6 cells incubated 24 h with 20 µM MG132, 100 ng/ml APO-1, or both, and then stained with Acridine Orange (AO). *Values* indicate the percentage of cells manifesting an abnormally low AO fluorescence (high lysosomal permeability). The graphics represent the mean ± SD from three different experiments. Differences among groups were statistically significant (*$p < 0.05$). **c** Western blotting for the lipidated form of LC3B protein (50 µg protein/lane) is indicated with an *arrow*. The electrophoresis was performed in 15% SDS-PAGE gels as described under "Materials and methods". Results are representative of three independent experiments

20 µM MG132 or with MG132 plus 100 ng/ml APO-1 (Fig. 3a, b) ($p < 0.05$). On the contrary, in the APO-1 and control group there is only a slight increase in lysosomal permeability (Fig. 3b), suggesting that the treatment with MG132 is mainly responsible for activating autophagy. Furthermore, we detected the lipidated form of LC3B through western blot in KE6 cells treated with MG132 and APO-1 plus MG132 in all analyzed times (Fig. 3c), which confirm that cell death is also activated by autophagy.

Treatment with APO-1 plus MG132 restores p53 stabilization

It is well known that the E6 oncoprotein induces a strong reduction in the level of the tumor suppressor p53 protein via ubiquitin-dependent proteolysis [4], and it has been reported that the proteasome inhibitor MG132 favors re-expression of p53 [19]. Because the effects of APO-1 and APO-1 plus MG132 on the p53 levels are unknown, we analyzed the expression of this protein through western blot at 3, 6, 12 and 24 h in KE6 cells treated with the previously mentioned compounds. As expected, our results show that MG132 treatment permit restoration of p53 levels (Fig. 4a). When both treatments were combined (APO-1 plus MG132) the expression level of p53 was similar to MG132 (Fig. 4a). Similar results were observed at 3, 6 and 12 h (Fig. 4b, c). At 24 h, p53 signal was almost lost in cells treated with MG132 and MG132 plus APO-1 (data not shown), probably as a consequence of protein degradation during apoptosis. Equal actin protein levels were observed in all treatments. On the other hand, it is known that several kinases bind and phosphorylate p53 in diverse regions during cellular stress. To determine whether p53 is phosphorylated at Ser20 or Ser46, which leads to the induction of apoptosis [21], we analyzed by western blot KE6 cells treated with APO-1 or MG132 or both compounds. We detected phosphorylation in p53

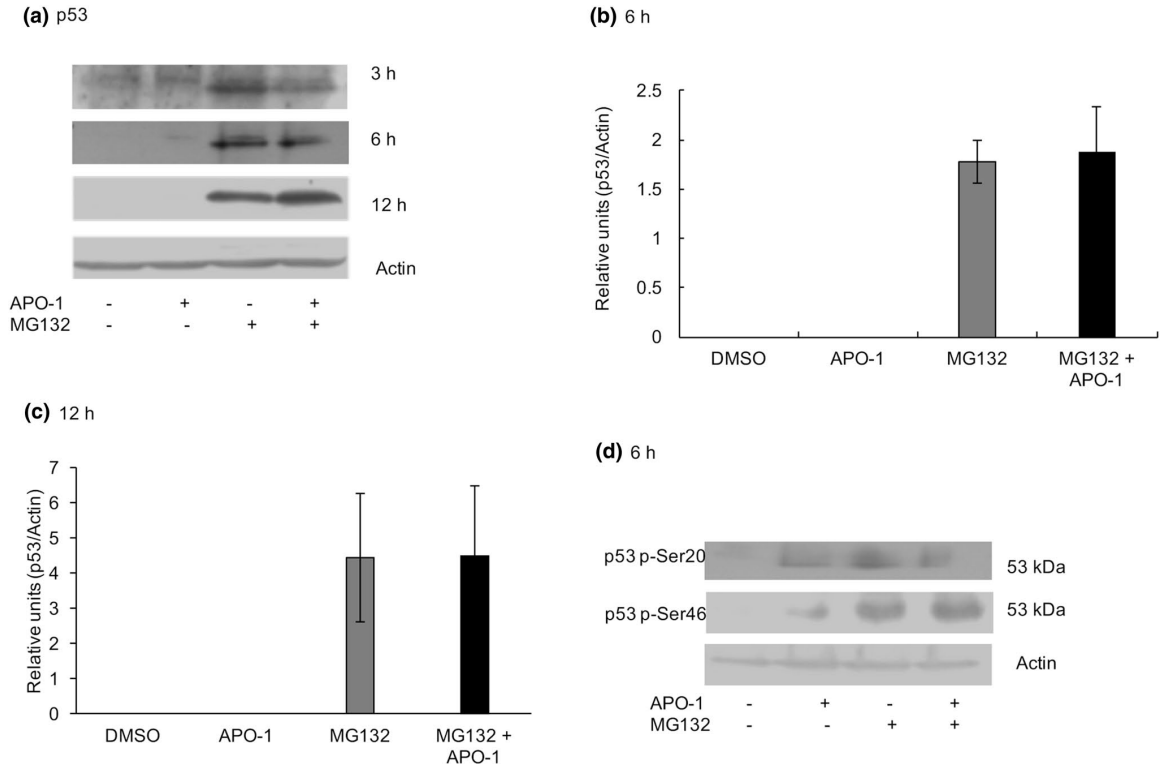

Fig. 4 Proteasome inhibition (MG132) causes stabilization of p53 and phosphorilation in Ser46 and Ser20 in KE6 cells. **a** Immunoblot for p53 using lysates (50 µg protein/lane) from KE6 cells treated for 3, 6 and 12 h with 20 µM MG132, 100 ng/ml APO-1, or both. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. The electrophoresis was performed in 10% SDS-PAGE gels as described under "Materials and methods". The data shown are representative of three independent experiments. **b**, **c** The graphics represent the mean of the densitometric analysis of p53. **d** Western blotting for p53 phosphorylation at Ser46 and Ser20 using lysates (50 µg protein/lane) from KE6 cells treated 6 h with 20 µM MG132, 100 ng/ml APO-1, or both

protein, specifically in Ser20 and Ser46 at 6 h (Fig. 4d), suggesting apoptosis induction. Interestingly, we observed a weak band in APO-1 compared to MG132 and APO-1 plus MG132 treatment but we do not know the importance of this result at this time. Similar results involving phosphorylation in p53 Ser46 were observed through confocal microscopy at 24 h (Online Resource 2).

During apoptosis induction p53 is localized in the nucleus

Accumulating evidence indicates that p53 protein can modulate apoptosis and autophagy in a dual fashion, depending on its posttranslational modifications and its subcellular localization. P53 functions as a nuclear transcription factor transactivating proapoptotic, cell cycle inhibitory and proautophagic genes. On the other hand, cytoplasmic p53 can operate at the mitochondria to promote apoptosis and repress autophagy [22]. Thus, we asked whether the increase in the p53 protein level that we detected by western blot results in nuclear or cytoplasmic localization and for this we examined its cellular localization in KE6 cells treated 6, 12 and 24 h with APO-1 or MG132, as well as with both compounds. Then, KE6 cells were fixed and tested with anti-human-p53 antibody and DAPI, as indicated in Materials and Methods. The results of confocal microscopy suggested that in MG132 or APO-1 plus MG132 treated cells, the signal of p53 was stronger in the nucleus (Fig. 5a). A weak signal of p53 protein was detected in the cytoplasm of KE6 cells treated with APO-1 (and in some cases in the nucleus). As expected, the p53 signal was detected only in a few KE6 control cells. The strong p53 signal in nucleus remains over time mainly in KE6 cells treated with MG132 and APO-1 plus MG132 (Fig. 5b). Due to the high cell death at 24 h with APO-1 plus MG132, we could only count approximately 40% of the total cells, in which p53 remained in all cases in the nucleus. These results suggest that p53 could be responsible for inducing cell death through the transcriptional induction of its target genes in MG132 and APO-1 plus MG132 treated cells.

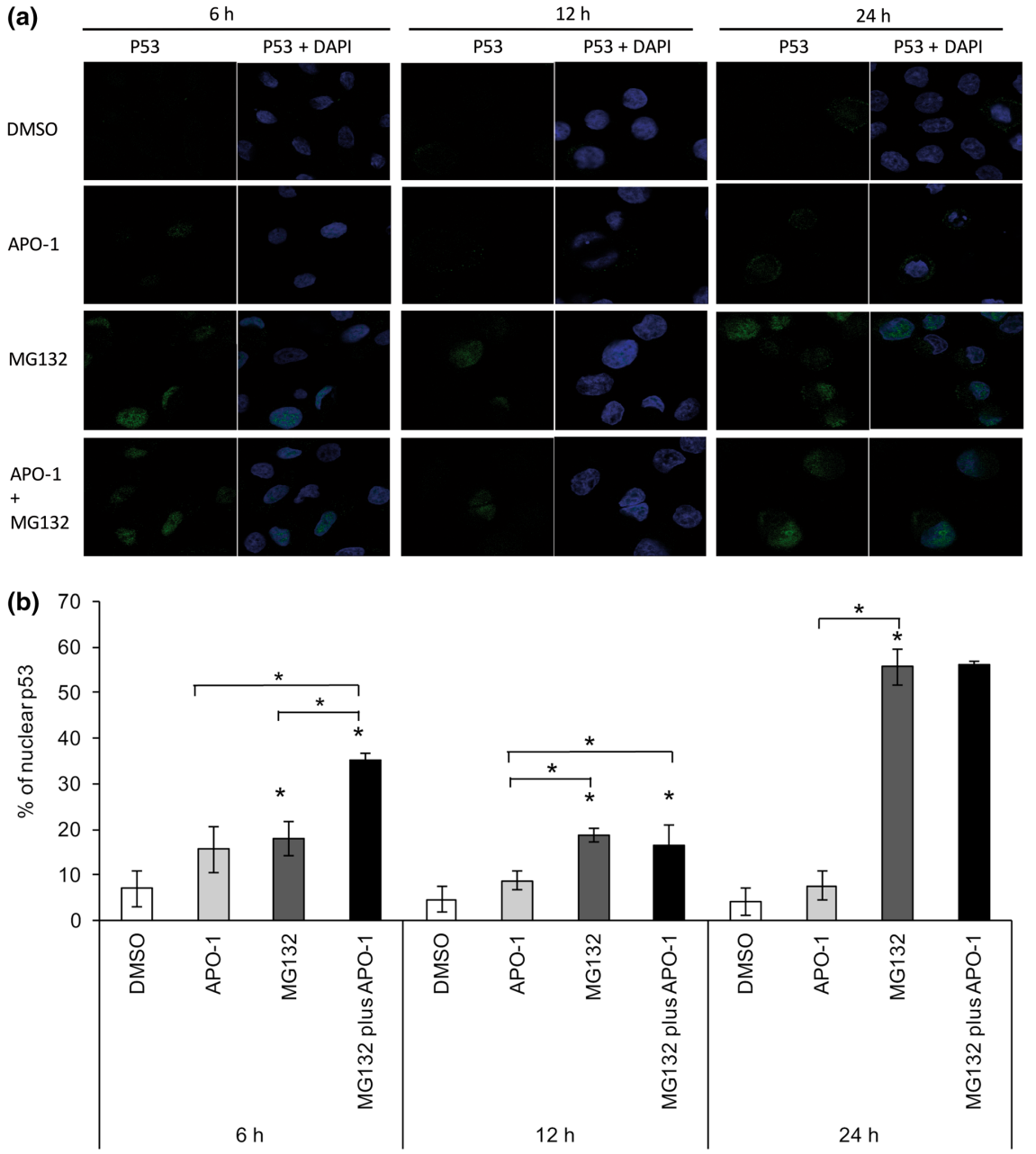

Fig. 5 MG132 and APO-1 treatments favors the nuclear accumulation of p53. **a** KE6 cells were stained for the detection of chromatin (DAPI, *blue* fluorescence) and p53 (*green* punctate staining). KE6 cells were incubated overnight at 4 °C with anti-p53 antibody, as indicated under "Materials and methods". Photomicrographs were taken 6, 12 and 24 h after treatment with 20 μM MG132, 100 ng/ml APO-1, or both. **b** Quantification of fluorescence signal of confocal microscopy experiments. One hundred cells were examined to detect p53 nuclear signal for each sample. The graphic is the result of this analysis. The graphics represent the mean ± SD from three independent assays (*$p < 0.05$). Statistical analyses were carried out between each treatment with the control (DMSO) or among the different groups

Restoration of high p53 levels by MG132 or APO-1 plus MG132 treatments modulates the expression of p53 target genes

After the inhibition of proteasome, in a short time (2–6 h) p53 induces transcription of its target genes, increasing the amount of corresponding mRNAs, which are translated to important proteins involved in cell cycle arrest, cell death and DNA repair. To identify whether the localization of p53 in the nucleus of KE6 cells after the different treatments is associated with transcriptional induction of p53 target genes, we analyzed the expression of genes involved in apoptosis (TP53INP1 and Bax) [23, 24], cell cycle (p21) [24] and autophagy (LC3). As shown in Fig. 6, all genes presented similar behavior after treatments. The expression of p21 was significantly increased in KE6 cells treated for 3 and 6 h with MG132 or APO-1 plus MG132; however, the highest increase in the level of p21 mRNA was observed after 6 h in cells treated only with MG132 ($p < 0.05$). Furthermore, in KE6 cells treated with APO-1 there was a decrease in the p21 gene expression observed at 3 h and a slight increase at 6 h. TP53INP1 mRNA levels were significantly increased at 3 and 6 h mainly in KE6 cells treated with MG132 or APO-1 plus MG132 ($p < 0.05$). Similar results were detected at 3 or 6 h for the expression of Bax mRNA level in KE6 cells treated with MG132 or APO-1 plus MG132. Besides, the LC3 mRNA expression presented a considerable increase at 6 h mainly in MG132 or APO-1 plus MG132. Interestingly, a statistically significant increase of LC3B and p21 mRNA levels were observed in the APO-1 treatment at 6 h ($p < 0.05$). These results showed induction of p53 target genes that participate in apoptosis and autophagy in MG132 and APO-1 plus MG132 treated cells.

Induction of apoptosis by MG132 and APO-1 treatments is dependent mainly on the p53 transcriptional activity but it does not require the c-Myc activity

To further explore the role of p53 activation in MG132 and APO-1-induced apoptosis, we investigated the effect of pifithrin-α (PFT-α), an inhibitor of p53-mediated apoptosis and p53-dependent gene transcription [25], on caspase-3 activity (as an indicator of apoptosis). We found that 2 h pre-incubation of KE6 cells with 10 µM PFT-α, significantly decreased caspase-3 activity in APO-1, MG132, or APO-1 plus MG132-treated cells compared to those without PFT-α (Fig. 7a) ($p < 0.05$). The reduction in caspase-3 activity was more evident for MG132 treated cells (3.7 fold) and APO-1 plus MG132 (three-fold) compared to APO-1 (2.8 fold). This result showed that p53 is an inductor of apoptosis in MG132 and MG132 plus APO-1 through caspase-3 activation.

As previously mentioned, c-Myc is a protein that is degraded by the E6 oncoprotein. Given that we also observed an increase in c-Myc protein levels in KE6 cells treated with MG132 and MG132 plus APO-1 at different treatments (Fig. 7b), we evaluated the role of c-Myc in MG132 plus APO-1-induced apoptosis. We observed a discrete but significant reduction in apoptosis in presence of 10058-F4 (an inhibitor of Myc/Max dimerization) (Fig. 7b).

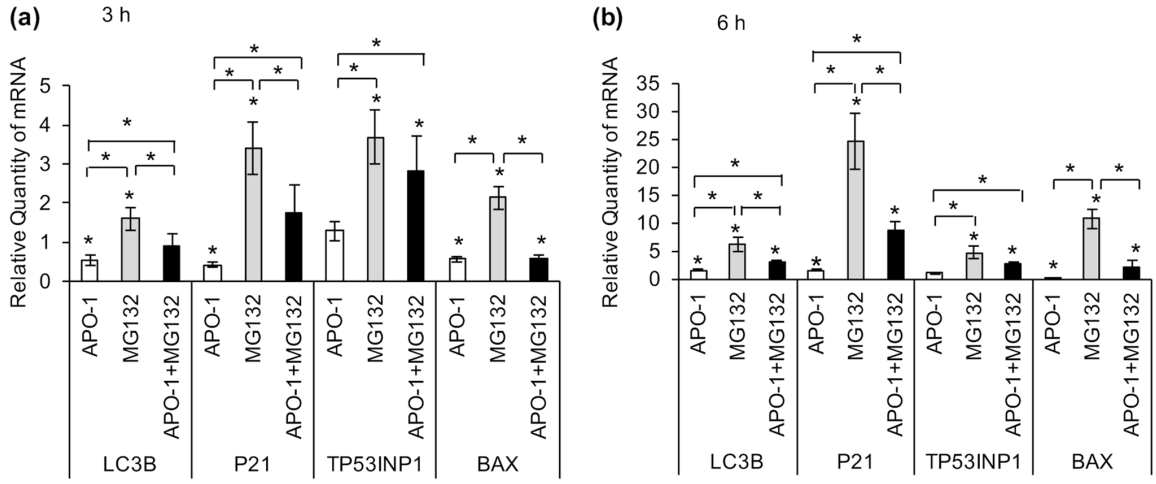

Fig. 6 Expression of p53 target genes in KE6 cells incubated with APO-1 and MG132, measured by RT-qPCR. KE6 cells were incubated with 20 µM MG132, 100 ng/ml APO-1, or both, for (**a**) 3 h or (**b**) 6 h. Experiment was performed as described under "Materials and methods". DMSO treated cells were used as calibrator for each gene tested. Data were analyzed with the equation: amount of target $= 2^{-\Delta\Delta CT}$ [22]. Mean ± SD for three independent experiments each performed in duplicate. Significant differences were found between treatments labeled with asterisk and control group (DMSO) or among the different groups, $^*p < 0.05$

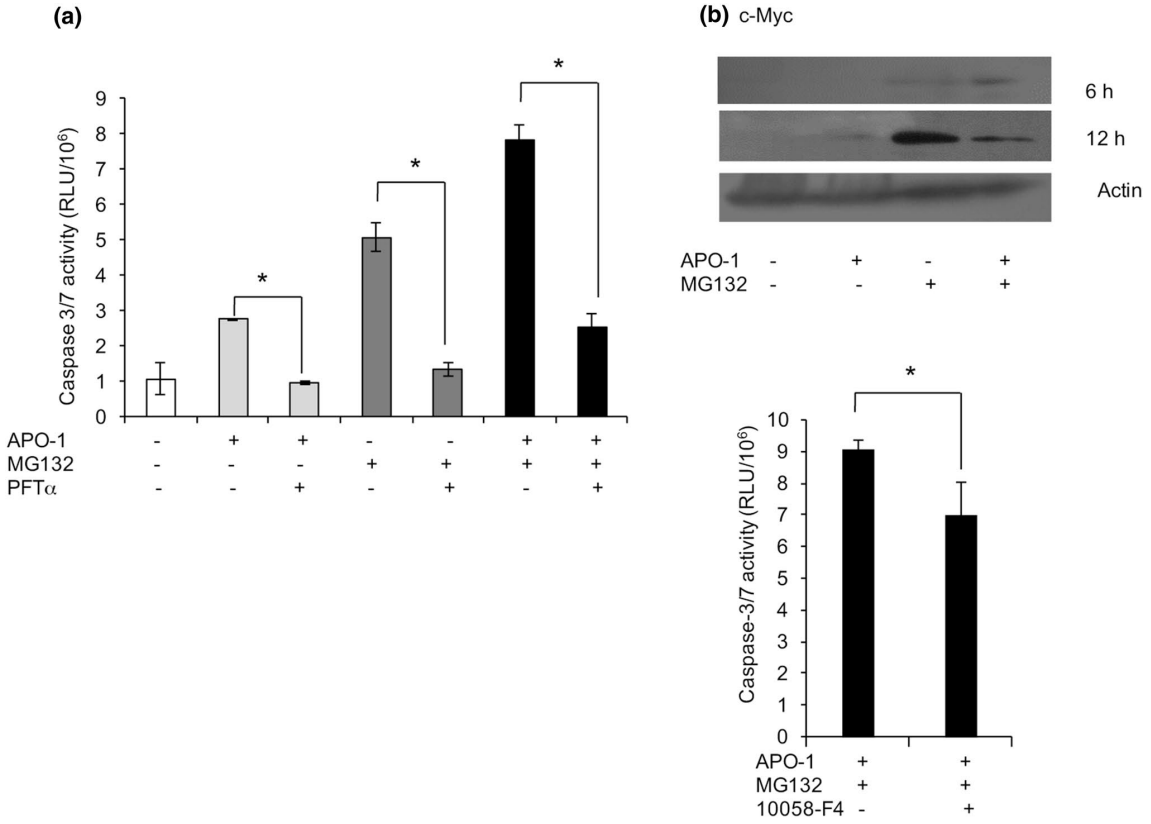

Fig. 7 Effect of pifithrin alpha (PFTα) and 10058-F4 on APO-1 and MG132-induced apoptosis. **a** KE6 cells were incubated 24 h with 100 ng/ml APO-1, 20 μM MG132, or APO-1 plus MG132, in the presence or absence of 10 μM pifithrin-α (an inhibitor of p53 activity). **b** Western blotting for c-Myc protein using lysates (50 μg protein/lane) from KE6 cells treated 6 and 12 h with 20 μM MG132, 100 ng/ml APO-1, or both. In the same conditions of PFT-α, KE6 cells were incubated with APO-1 plus MG132 in the absence or presence of 10058-F4 (an inhibitor of Myc/Max dimerization). Caspase-3/7 activity was determined by hydrolysis of the luminogenic substrate containing the DEVD sequence. Readings were taken 0.5 h after adding the caspase reagent; luminescence is proportional to caspase-3/7 activity expressed as RLU. The no-cell blank control value has been subtracted from each sample. Each *point* represents average of triplicates. Significant differences were found between APO-1 plus MG132 vs. APO-1 plus MG132 plus 10058-F4, $*p < 0.05$

This result suggests a weak role for c-Myc in MG132 plus APO-1-induced apoptosis compared to p53.

We think that the transcriptional activity of p53 is an important player in the induction of APO-1 and MG132-mediated apoptosis, which is observed by the reduction of caspase-3 activity (a primary executer of apoptosis) under the different treatments.

Discussion

In this study, we found that the restoration of both p53 expression and activity mediated by MG132 and APO-1 favors the induction of apoptosis and autophagy in KE6 cells; after treatment we localized the p53 protein inside the nucleus and demonstrated that several p53 targets are transcriptionally activated. Previously, it was suggested that restoration of p53 expression sensitizes HPV16 E6 immortalized human keratinocytes (KE6 cells) to CD95-mediated apoptosis but blockage of proteasomal activity alone in a short treatment time did not result in apoptosis [19]. However, we found that MG132 treatment alone is sufficient to induce a high level of apoptosis, which is mostly dependent on p53 transcriptional activity (inhibited by PFT). To verify this effect, we performed an MTT assay of cervical cancer cell lines (HeLa and SiHa), KE6 cells and normal keratinocytes (NK). Treatment for 24 h of the above cell lines with 20 μM MG132 and MG132 plus 100 ng/ml APO-1, but not 100 ng/ml APO-1 alone induced growth inhibition of HeLa (35 and 81%), SiHa (45 and 41%), KE6 (49 and 89%) and NK (31 and 67%). KE6 was the most sensitive cell line, perhaps because in the absence of E7, p53 inhibition by E6

oncoprotein is critical for the inhibition of apoptosis (Online Resource 3).

It has been demonstrated that HR-E6 degrades several proapoptotic proteins (FADD, TNF-R1, procaspase-8, Bax and Bak) [3], inhibiting apoptosis. According with the inactivation of proapoptotic proteins by HR-E6, we and others [19, 26] think that it is necessary to first block the proteasome complex activity (for example with MG132) to restore proapoptotic protein levels, and then induce the activation of the extrinsic apoptosis pathway with Fas ligand or an antibody that imitates the action of Fas ligand or TNF-alpha. Because p53 induces apoptosis mainly through the intrinsic pathway we suggest that this pathway is the main factor in the induction of apoptosis mediated by MG132 plus CD95 possibly via cleavage of Bid. Consistent with our work, it was reported that MG132 sensitizes cervical cancer cell lines (HeLa and SiHa) to TRAIL-induced apoptosis; unfortunately autophagy or p53 phosphorylation was not studied in this case [26]. Besides, it has been demonstrated that in the presence of TRAIL, human osteosarcoma cells exhibited a low apoptosis rate [27]. In contrast, MG132 alone and MG132 plus TRAIL dramatically augmented apoptosis in human osteosarcoma and glioma cells [27, 28]. These results suggest that in Fas or TRAIL pathways, proteasome inhibitors (PI) may be necessary in addition to the ligand for extrinsic apoptosis induction. Regarding the intrinsic apoptosis pathway, we found that the mitochondrial membrane potential was decreased by MG132 alone and particularly by APO-1 plus MG132 (Fig. 2b). The decrease in the mitochondrial membrane potential could be explained by the activation of the p53 pathway through MG132, in addition to the stabilization and activation of other cellular proteins, which are required for a robust apoptosis induction; however, the experiments with PFT and 10058-F4 (an inhibitor of Myc/Max dimerization) suggest that p53 plays a major role in intrinsic apoptosis.

It is known that E6 binds to FADD and protects cells from CD95 triggered apoptosis [6], which prevents caspase-8 and caspase-3 activation. Our findings show that APO-1, MG132 and APO-1 plus MG132 treatments induce the activation of caspase-8 in KE6 cells (Fig. 1c). Also, under the same experimental conditions employed for caspase-8 activation described in our work, we also observed caspase-9 activation (Fig. 1d) suggesting again that even in the presence of E6, all treatments are able to induce both intrinsic and extrinsic apoptosis in varying proportions.

We think that in addition to the activation of the intrinsic pathway by the restoration of p53, in KE6 cells treated with APO-1 and MG132, a slight caspase-8 activation level could mediate Bid cleavage to generate the active truncated form (tBid) and cooperate to promote the intrinsic mitochondrial pathway [29]. In relation with this report, we observed a decrease in the full-length Bid protein levels, mainly when the treatment with APO-1 plus MG132 was used. This result suggests that Bid transmit an apoptotic signal from Fas receptor to the mitochondria strengthening the activation of the intrinsic pathway.

In cervical cancer a few reports have suggested HPV-mediated autophagy inhibition [30, 31], but the mechanism through which HPV oncoproteins inhibit autophagy is unknown. Interestingly, our results in KE6 cells show induction of autophagy by MG132 and mainly by APO-1 plus MG132 (Fig. 3), which is consistent with increased levels of p53 protein. To date it is unknown whether APO-1 induces autophagy by itself, but here we show a cooperation of APO-1 with MG132 to increase the induction of autophagy. We observed lipidation of LC3B through western blot confirming that treatments with MG132 and APO-1 plus MG132 in KE6 cells favor activation of autophagy.

Autophagy can be part of the cascade of events that lead to cell death, either by collaborating with other cell death mechanisms or by causing cell death on its own [8]. Our results show 3 times more apoptosis induction than autophagy in KE6 cells treated with APO-1 plus MG132 (Figs. 1b, 3b). This result may suggest the importance of apoptosis in the cell death mediated by APO-1 and MG132 as compared to the minor contribution by autophagy. We thought that MG132 could stabilize some unknown proteins that APO-1 needs to increase p53 levels. In this study we demonstrated that nuclear p53 functions as a proapoptotic and/or proautophagic transcription factor. In addition, it has been reported that cytoplasmic p53 suppresses autophagy in a number of experimental settings, and that the inactivation of p53 can induce autophagy [32]. These results demonstrated a direct relation between p53 cellular localization and activation/inhibition of autophagy. Thus, our observations suggest that nuclear p53 can induce cell death mainly mediated by apoptosis with a minor autophagy contribution.

P53 is normally a short-lived protein, maintained at low levels in unstressed mammalian cells [33]. Following stress, p53 becomes stabilized and activated through extensive posttranslational modification, such as phosphorylation in Ser15 and Ser20 [34]. Phosphorylation of Ser46 is the earliest and perhaps the most clear example of a modification in p53 that is critical for p53-mediated induction of proapoptotic genes. We observed for the first time a weak phosphorylation in p53 Ser20 and Ser46 after APO-1, MG132 and APO-1 plus MG132 treatments of KE6 cells suggesting that extrinsic and/or intrinsic apoptosis can induce both high p53 levels and phosphorylation on several p53 serines. The biological implications of this posttranslational modification are been evaluated in our group.

Phosphorylation in p53 Ser46 correlates with results observed in Fig. 6, in which we demonstrated that p53 reactivation retained the ability to induce proapoptotic target genes, such as p21 [35], Tumor Protein 53-Induced Nuclear

Protein 1 (TP53INP1) [36] and Bax. Similar to our work, Gareau et al., found that p21 mRNA is induced in HeLa cells by Bortezomib (another proteasome inhibitor) at 4 and 10 h [37].

Comparable to p21, TP53INP1 mRNA increases at 3 h and at 6 h in KE6 cells treated with MG132 or APO-1 plus MG132. Since TP53INP1 promotes autophagy [38, 39], it is possible that these conditions also favor autophagy [39]. Furthermore, it has been reported that TP53INP1 phosphorylates p53 protein at Ser46 enhancing its stability and promoting the binding of p53 to the promoter regions of proapoptotic genes, rather than to those of repair-related genes [40].

Our results on the expression of proapoptotic Bax gene are in accord to those of Ortiz-Lazareno et al. showing that the expression of Bax mRNA was increased in U937 cells treated with MG132 and MG132 plus Doxorubicin compared with untreated cells [41]. The LC3B lipidation and the increase of LC3B mRNA, mainly in KE6 cells treated with MG132 and MG132 plus APO-1 demonstrate the activation of autophagy.

We have observed that apoptosis induced in KE6 cells after the individual or the combined treatments is mainly dependent on the transcriptional activity of p53 because 10 μM PFT-α suppressed this process (Fig. 7a); similar results were obtained using 30 μM PFT-α (Online Resource 4). We observed a 78% decrease in the activity of caspase-3/7 when KE6 was treated with APO-1 plus MG132 plus PFT-α (Fig. 7a). This result makes clear the importance of the p53 transcriptional activity in the intrinsic apoptosis pathway. However, the remaining 22% of activity of these caspases might be due to the activation of p53-independent pathways. For this reason, we evaluated the possible role of c-Myc in MG132 plus APO-1-induced apoptosis. We demonstrated that c-Myc has a discrete role in apoptosis induced by these compounds. At this time we ignore the role of others members of the p53 family as p73 in the MG132 plus APO-1-induced apoptosis.

In conclusion, we demonstrate that MG132 and APO-1 cooperate to restore p53 activity and induce autophagy and p53-dependent apoptosis in E6-expressing keratinocytes. Thus, the utility of APO-1 in combination with this proteasome inhibitor could prove to be a cervical cancer therapeutic strategy.

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Compliance with ethical standards

Conflict of interest The authors declare that have no conflicts of interest.

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Protein oligomerization mediated by the transmembrane carboxyl terminal domain of Bcl-XL

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ABSTRACT

Bcl-XL is a pro-survival member of the Bcl-2 family that can be found in the outer mitochondrial membrane and in soluble cytosolic homodimers. Bcl-XL can bind pro-apoptotic members of this family preventing them from activating the execution phase of apoptosis. Bcl-XL has been shown to homodimerize in different ways, although most binding and structural assays have been carried out in the absence of its carboxyl terminal transmembrane domain. We show here that this domain can by itself direct protein oligomerization, which could be related to its previously reported role in mitochondrial morphology alterations and apoptosis inhibition.

Structured summary of protein interactions:
Vamp2 physically interacts with **Vamp2** by blue native page (View interaction)
Vamp2 physically interacts with **Vamp2** by cross-linking study (View interaction)
Bcl-Xl physically interacts with **Bcl-Xl** by blue native page (View interaction)
Bcl-Xl physically interacts with **Bcl-Xl** by cross-linking study (View interaction)

1. Introduction

Apoptosis is a type of cell death characterized by the silent elimination of unnecessary or damaged cells, which is required for development and tissue homeostasis. Two major routes of apoptosis have been described: the extrinsic route is initiated at the cell surface and mediated by death receptors, like Fas-CD95 [1]; the intrinsic route is mediated by mitochondria [2], where many different signals are integrated and the final decision about the fate of the cell is made. Both routes are linked in some cells by Bid, a member of the Bcl-2 protein family [3]. Bcl-2 family members participate directly in the decision step [4]. Pro-apoptotic members of this family, Bax and Bak, have been shown to permeabilize the mitochondrial outer membrane (MOM), facilitating the release to the cytosol of intermembrane space (IMS) proteins like cytochrome c [5], AIF [6] or Smac/DIABLO [7,8]. These proteins then activate a series of caspase-dependent or -independent mechanisms that dismantle the cell. A second mechanism for the release of apoptotic factors from the IMS involves permeability transition, which allows the entry of water in the matrix with subsequent burst of the MOM [9].

A complex interplay between pro-apoptotic and pro-survival Bcl-2 family members controls MOM permeabilization (MOMP), considered the point-of-no-return during apoptosis induction. Several models have been proposed to explain how Bcl-2 members regulate MOMP through interactions between pro-survival and pro-apoptotic proteins, both in solution and at the MOM [10]. Activation of these proteins involves conformational changes, specially relevant for Bax, which usually resides in the cytosol and requires a conformational change to expose hydrophobic domains required for membrane insertion, followed by further changes necessary for pore formation through oligomerization at the MOM [11].

Homodimerization of the pro-survival protein Bcl-XL in the cytosol involves a C-terminal membrane-targeting α helix from one monomer and a hydrophobic groove on the other monomer [12], although other dimerization modes have been described [13–16]. Release of that helix from the hydrophobic groove allows its insertion into the MOM. Most interaction and structural studies

Abbreviations: PSAP/Mtch1, presenilin 1-associated protein/mitochondrial carrier homolog 1; MOM, mitochondrial outer membrane; IMS, intermembrane space; MOMP, mitochondrial outer membrane permeabilization; TMD, transmembrane domain; mRFP, monomeric red fluorescent protein; VAMP2, vesicle-associated membrane protein 2

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carried out with this protein employed deletion mutants lacking the C-terminal helix. This mutant can also bind membranes through its N-terminal domain [17], and Bax, Bcl-2 and Bcl-XL have been reported to insert α-helices 5 and 6 into the MOM during apoptosis [18–20]. Many interaction and structural studies have been carried out in the absence of membranes, underestimating their role in protein interactions and conformational changes [17,21].

Since the C-terminal helix (transmembrane domain or TMD) of Bcl-XL and flanking sequences contain the necessary information for MOM targeting and insertion in a C-in N-out orientation [22], we used this domain to target fragments of PSAP/Mtch1 to the outer mitochondrial membrane [23]. PSAP was first identified as a presenilin-1 associated protein with homology to inner membrane mitochondrial carriers, and therefore is also known as mitochondrial carrier homolog 1 (Mtch1) [24]. The importance of its closest homolog, Mtch2, in Bid-induced apoptosis has been reported recently [25]. We reported that PSAP is a MOM protein with two pro-apoptotic domains [23]. Since apoptosis induction by these domains could depend on interactions with other proteins, we sought to analyze PSAP interactions by crosslinking. These assays suggested that the TMD of Bcl-XL could be involved in oligomerization, which was confirmed analyzing fusions to monomeric red fluorescent protein (mRFP).

2. Materials and methods

2.1. General reagents

All reagents were of molecular biology grade. Restriction enzymes were from Roche, Stratagene, Invitrogen, Fermentas and New England Biolabs; Accuprime Pfx DNA polymerase, custom-made primers and T4 DNA Ligase were from Invitrogen; Pfu polymerase, from Stratagene.

2.2. Construction of expression vectors

Expression vectors containing PSAP or Mtch2 sequences have been previously described [23]. pJAC295, expressing myc-mRFP-TMD Bcl-XL (39.1 kDa), was constructed by amplifying the mRFP sequence from another vector by PCR with primers containing Eco RI (mRFPEcoF: 5′-CTAGGATCGAATTCGGATGGCCTCCTCCGAGGACGT-3′) and Hind III (mRFPHindR: 5′-CTAGGATCAAGCTTGGC GCCGGTGGAGTGGCGGC-3′) restriction sites. The digested PCR product was used to replace PSAP sequences in the vector expressing myc-PSAP65-112-Bcl (which contains the TMD of Bcl-XL) [23]. The resulting vector expresses mRFP preceded by a myc tag and followed by the sequence KLESRKGQERFNRWFLTGMTVAGVVLL GSLFSRK, where the first two amino acids (kl) correspond to the Hind III site and the remaining amino acids, to the TMD of Bcl-XL.

The same approach was used to construct vector pAOC2, expressing myc-mRFP-TMD VAMP2 (32.2 kDa), replacing PSAP sequences in a vector that expresses myc-PSAP39-168-Vamp (which contains the TMD of VAMP2) [23]. In this case, sequence KLLKRKYWWKNLKMMIILGVICAIILIIIIVYFSS followed mRFP. pAL2 (28.7 kDa), expressing myc-mRFP, was constructed by digesting pJAC295 with Hind III and Not I, to eliminate the TMD of Bcl-XL, blunting with Pfu polymerase, and re-ligating. Vectors were transformed into *E. coli* JM109 from Promega. Correct clones were confirmed by restriction digestion and sequencing. pAOC5, expressing myc-Bcl-XL (full length Bcl-XL preceded by a myc tag, 28.6 kDa), was constructed by carrying out a PCR on a vector containing the cDNA for Bcl-XL with primers BCLXLECOF (5′-CTAGGATCGAA TTCGAATGTCTCAGAGCAACAACCGGGAGC-3′) and BLCLXLNOTR (5′-CTAGGATCGCGGCCGCTCATTTCCGACTGAAGAGTGAG-3′). The PCR product was digested with Eco RI and Not I and inserted into pCMVMyc. pAOC6, expressing myc-BclXL-ΔTMD (Bcl-XL without its carboxyl terminal transmembrane domain, preceded by a myc tag, 26.7 kDa), was constructed by PCR with primers BCLXLECOF and BCLXLWOTMXHOR (5′-CTAGGATCCTCGAGGGCTGCTGCATT GTTCCCATAG-3′). The product was digested with Eco RI and Xho I and cloned into pCMVMyc. pAOC7, expressing myc-BclXL-TMD2Pit2 (BclXL preceded by a myc tag and with its carboxyl terminal domain replaced by the second transmembrane domain of Pit 2, 27.3 kDa) was constructed by doing PCR on vector pVLG60 (pEGFpN1-PSAP-DTM1-Pit2TM2, see Lamarca et al. [26]) with primers Pit2TM2XhoF (5′-CTAGGATCCTCGAGAGGCAGGCATGCA TTTTAGCTTC-3′) and Pit2TM2NotR (5′-CATGGTACGCGGCCGC TCATTTGGCGCCTAGTAACACGGAG), the product was digested with Xho I and Not I and used to replace the equivalent fragment in vector pAOC6. The sequence of the second transmembrane domain of Pit 2 (TMD2Pit2) used is RQACILASIFETTGSVLLGAK. pAOC8, which expresses myc-BclXL-TMD3Pit2 (BclXL preceded by a myc tag and with its carboxyl terminal domain replaced by the third transmembrane domain of Pit 2, 27.3 kDa), was generated by PCR with primers Pit2TM3XhoF (5′-CTAGGATCCTCGAGATGGCTGGGGAAGTT AGTGC-3′) and Pit2TM3NotR (5′-CTAGGATCGCGGCCGCTCACAGG AAGGAAGCAATCAGCT-3′) in the same way as done with pAOC7, but using vector pVLG61 (pEGFpN1-PSAP-DTMI-Pit2TM3, see Lamarca et al. [26]) as template for the PCR. The sequence of the third transmembrane domain of Pit2 (TMD3Pit2) used is MAGEVSAMVGSAVWQLIASFL.

2.3. Cell culture and transfection

HEK293 cells and HeLa cells were cultured as previously described [26] and transfected using GeneJuice Reagent (Novagen) at a confluence of 80% in 24-well plates.

2.4. Crosslinking and immunoblotting

12 h post-transfection, cells were washed twice with 500 μl phosphate-buffered saline (PBS) and resuspended in 180 μl crosslinking buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.5) containing 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF). To detect de effect of expression of chimeras on Bax and Bak oligomerization, 12 h post-transfection cells were treated with 30 μM camptothecin for 24 h, and then processed as indicated. Samples were divided in two tubes, 10 μl of a 25 mM solution in DMSO of the amine-reactive membrane-permeant crosslinker BSOCOES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone) (Pierce) was added to one tube and 10 μl DMSO to the second tube, and incubated for 1 h or 20 min (for Bax and Bak) at room temperature with agitation. 5 μl quenching solution (1 M Tris Hcl, pH 7.5) was then added and samples agitated for 15 min at room temperature. Cells were recovered by centrifugation and analyzed using SDS–PAGE (Laemmli) followed by immunoblotting with anti-myc (Invitrogen), anti-PSAP MGAS [26], anti-Bax (Millipore) or anti-Bak (Millipore) antibodies after transfer onto polyvinylidene fluoride (PVDF) membranes (Invitrogen), as described [23]. Proteins were visualized by incubation with goat-anti mouse or goat anti-rabbit secondary antibodies conjugated to HRP and detected by ECL. Molecular mass markers were from Invitrogen (BenchMark Pre-Stained Protein Ladder) or Bio-Rad (Precision Plus Protein Standards).

2.5. Blue-native electrophoresis (BN-PAGE)

BN-PAGE was carried out with digitonin-treated cells using the NativePAGE™ Novex® Bis–Tris Gel System from Invitrogen, 4–16% gradient gels, following instructions supplied by the manufacturer. 10 μg protein was loaded per well. Native protein standards were from Invitrogen (NativeMark Unstained Protein Standard).

2.6. Cell viability and death assays

We used the Cell Proliferation Kit II (XTT) (Roche) as a quick method to measure cell death induced by camptothecin. HEK293 cells were cultured and transfected in 96-well plates, 12 h post-transfection cells were treated with 30 μM camptothecin or DMSO and 24 h later they were incubated with XTT. Two independent assays were carried out in triplicate.

For trypan blue exclusion assays, HEK293 cells were cultured in 24-well plates until they reached 80% confluency and then transfected and treated with camptothecin or DMSO as indicated above. 24 h post-transfection wells were washed with PBS, which was transferred to tubes to avoid loosing detached cells. Attached cells were trypsinized for 5 min and mixed with detached cells in PBS from the same well. Complete media, with serum, was added to inactivate trypsin. Cells were mixed with trypan blue (Roche) in a 1:1 ratio and counted using a hemacytometer. For each sample, four quadrants were scored twice, with cell numbers ranging between 18 and 35 per quadrant. For one of the experiments an automatic cell counter (Countess from Invitrogen) was used, obtaining similar results as with manual counts. Each experiment was carried out in triplicates three independent times.

2.7. Immunocytochemistry

For subcellular localization of Bcl-XL chimeras, cells grown on round coverslides in 24-well plates were first incubated with 25 nM Mitotracker Red CMX-Ros (Invitrogen) for 30 min at 37 °C, the culture media replaced with fresh media and incubated again for 30 min at 37 °C. Cells were then fixed with a solution containing 50% culture media and 50% of a 3.7% formaldehyde solution in

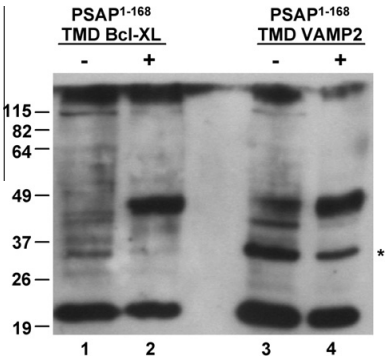

Fig. 1. Crosslinking of PSAP chimeras. Western blot of crosslinker-treated cells (+) or untreated (−) expressing PSAP1-168-TMD Bcl-XL or PSAP1-168-TMD VAMP2, separated on a 10% SDS gel and detected with anti-MGAS. Sizes of molecular mass markers are indicated on the left. Bands likely corresponding to heterodimers of PSAP1-168-TMD VAMP2 with endogenous VAMP2 are indicated with an asterisk on the right.

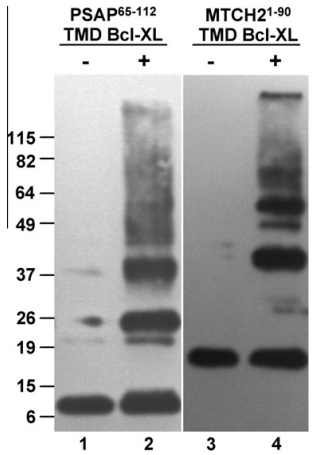

Fig. 2. Crosslinking of PSAP and Mtch2 chimeras. Western blot of crosslinker-treated cells (+) or untreated (-) expressing myc-PSAP-65-112-TMD Bcl-XL or myc-Mtch2-1-90-TMD Bcl-XL, separated on a 10% SDS gel and detected with anti-myc. Sizes of molecular mass markers are indicated on the left.

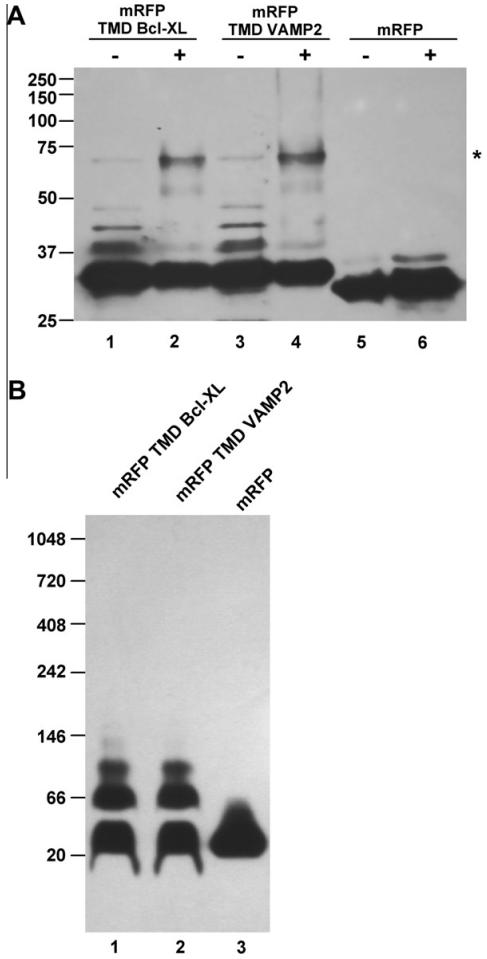

Fig. 3. Crosslinking and blue-native electrophoresis of mRFP chimeras. (A) Western blot of crosslinker-treated (+) or untreated (−) cells expressing myc-mRFP-TM Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone, separated on a 10% SDS gel and detected with anti-myc. Sizes of molecular mass markers are indicated on the left. Bands corresponding to dimers (63.7 and 64.4 kDa) are indicated with an asterisk on the right. (B) Blue-native electrophoresis analysis of mRFP chimeras. Digitonin-solubilized proteins from cells expressing myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone were separated on native 4–16% gradient gels, blotted and detected with anti-myc. Sizes of native molecular mass markers are indicated on the left.

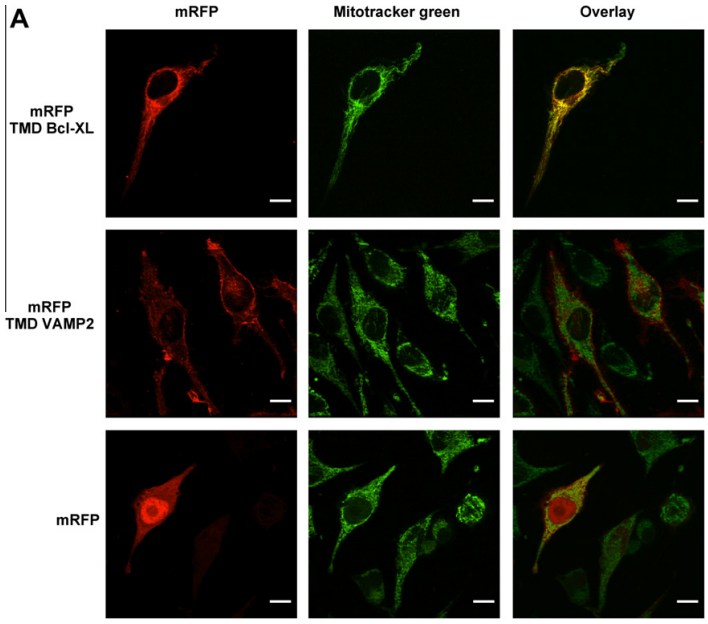

Fig. 4. Subcellular localization of mRFP and Bcl-XL chimeras. (A) HeLa cells were grown on round coverslips, transfected with vectors encoding myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP, incubated with Mitotracker green, fixed with formaldehyde and mounted for microscopy. (B) HeLa cells grown as in A and transfected with myc-BclXL, myc-BclXL-ΔTM, myc-BclXL-TMD2Pit or myc-BclXL-TMD3Pit, incubated with Mitotracker red CMX-Ros, with an anti-myc antibody, with goat anti-mouse Alexa fluor 488-conjugated antibody and mounted for microscopy. Images were acquired with a Leica DMI6000B inverted fluorescence microscope using structured illumination (Optigrid) with filters suitable for mRFP/Mitotracker Red and for Mitotracker green/Alexa fluor 488, and overlayed to show co-localization using software Metamorph. Bar is 10 μm.

PBS for 5 min, followed by a 15-min incubation with 3.7% formaldehyde solution in PBS, and then blocked and permeabilized with a solution containing 1% BSA and 0.1% TX-100 in PBS for 1 h. After three 10-min washes with PBS, cells were incubated with an anti-myc antibody from Invitrogen at a 1:700 dilution for 2 h, washed with PBS and incubated with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1 h, washed again with PBS and mounted for microscopy using Fluoromount G (Sourthernbiotech).

Subcellular localization of mRFP chimeras was carried out as for Bcl-XL chimeras but using 100 nM Mitotracker Green.

2.8. Microscopy

We used a Leica DMI6000B inverted fluorescence microscope with structured illumination (Optigrid) and software Metamorph. For red fluorescent protein and Mitotracker red we used a BP560/40 excitation filter and a BP645/75 emission filter with a 595 nm dicroic, and for Mitotracker green and Alexa Fluor 488, we used a filter for GFP, excitation BP470/40, emission BP525/50 and 500 nm dicroic.

3. Results

In order to study PSAP function we generated several deletion mutants which were used to study mitochondrial import and induction of apoptosis by this protein [23]. We targeted PSAP fragments to the MOM by fusion to the TMD of Bcl-XL, demonstrating that MOM localization was required for apoptosis induction, since endoplasmic reticulum-targeting by fusion to the TMD of VAMP2 [27] did not induce apoptosis.

Since apoptosis induction by PSAP could be mediated by interactions with other proteins, we attempted to detect PSAP-interacting proteins by crosslinking, using some of the above-mentioned chimeras. When HEK293 cells expressing the first 168 residues of PSAP fused to the TMD of Bcl-XL ($PSAP^{1-168}$TMD Bcl-XL, 21 kDa) were treated with BSOCOES, a band was observed by western blot suggesting dimerization of the protein (Fig. 1, lane 2). Similar results were obtained with the protein containing the TMD of VAMP2 ($PSAP^{1-168}$TMD VAMP2, 21.2 kDa). Since both proteins share the same PSAP sequences, we first assumed that PSAP was mediating dimerization. Nevertheless, a literature search indicated that the TMD of VAMP2 is responsible for its homo or heteromerization at the ER membrane [28], suggesting that the dimers observed in our experiments could depend on TMD instead of PSAP sequences. This interpretation could also explain a band migrating between those corresponding to the monomer (21.2 kDa) and dimer (42.4 kDa) in lanes 3 and 4 of Fig. 1, as a heterodimer between endogenous VAMP2 (12.6 kDa) and $PSAP^{1-168}$TMD VAMP2 (expected molecular mass: 33.8 kDa, marked with an asterisk in Fig. 1). Note that some of the bands are also present in the absence of crosslinker, suggesting strong interactions.

When similar constructs containing shorter PSAP fragments were used, oligomerization was observed. Fig. 2 shows the results obtained with a construct containing residues 65–112 preceded by a myc tag and followed by the TMD of Bcl-XL (11.2 kDa). Crosslinking of a protein chimera containing Mtch2 sequences (16 kDa) instead of PSAP sequences (lanes 3 and 4 in Fig. 2) also produced oligomers.

Our constructs contained parts of PSAP or Mtch2 and the TMD of Bcl-XL, proteins that can all be inserted into the MOM, therefore we could not rule out a mixed effect due to different sequences from either protein. In fact, whether dimers or oligomers were observed depended on the sequences attached to the TMD (Fig. 1 and Fig. 2). In order to clarify this subject, we fused the TMD of Bcl-XL or that of VAMP2 to monomeric red fluorescent protein

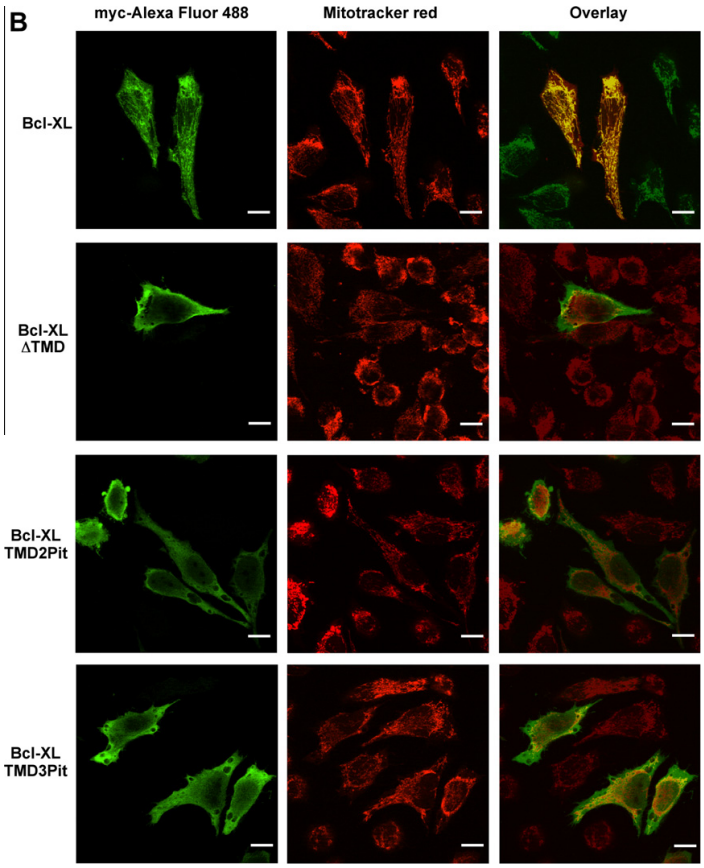

Fig. 4 (*continued*)

(mRFP) preceded by a myc tag for detection. We used myc-mRFP (28.7 kDa) as control. Crosslinking assays indicated that similar complexes could be detected when each TMD was fused to mRFP (Fig. 3A), whereas myc-mRFP remained as a monomer, therefore indicating that the TMDs were responsible for the dimers observed. The contribution of PSAP sequences to dimers and oligomers shown in Fig. 1 and 2 is under investigation and will be reported elsewhere.

In order to analyze these interactions with a different technique, we used blue-native electrophoresis (Fig. 3B), confirming that fusion of mRFP to either the TMD of Bcl-XL or the TMD of VAMP2 induced oligomerization of the protein. The pattern obtained indicated the presence of oligomers up to pentamers, although less abundant (or less stable) than the dimers. These data clearly indicate the involvement of the TMD of Bcl-XL in self-association, an event that had not been reported previously. The fact that only dimers could be observed upon crosslinking could be due to the location of the reacting amino groups in the oligomers. Crosslinking covalently fixes those interactions where reacting groups (amino groups in this case) are localized in close proximity, and uncrosslinked molecules are separated later during denaturing electrophoresis. Blue native electrophoresis does not rely on specific reactive groups but on the overall stability of protein complexes where individual molecules are held together by non-covalent bonds. It could be that, in our complexes, reacting amino groups are only located closely between monomers of a single dimer, and dimers associate in oligomers in such a way that adjacent dimers cannot be crosslinked with each other. This could explain why dimers are mainly observed upon crosslinking whereas oligomers are observed after blue-native electrophoresis.

We analyzed the subcellular localization of our chimeras by fluorescence microscopy, using Mitotracker green to label mitochondria. As expected, mRFP-TMD Bcl-XL localized to mitochondria, mRFP-TMD VAMP2 localized to membranes and mRFP distributed evenly throughout the cell (Fig. 4A).

The TMD of Bcl-XL fused to yellow fluorescent protein has been reported to alter mitochondrial morphology and to moderately protect cells against staurosporine-induced apoptosis [29]. In order to find out if our mRFP-TMD Bcl-XL chimera also protected cells against camptothecin-induced cell death we carried out cell viability assays using XTT, which were later confirmed by trypan blue exclusion assays. The results, shown in Fig 5, clearly indicated that the TMD of Bcl-XL fused to mRFP protected cells from death, whereas the TMD of VAMP2 fused to mRFP or mRFP alone did not.

Since Bcl-XL can prevent Bax and Bak oligomerization, we analyzed the state of oligomerization of these two proapoptotic proteins upon transfection of cells with mRFP chimeras and induction of apoptosis with camptothecin. We did not see any effect on Bax and Bak oligomerization in cells transfected with either mRFP-TMD Bcl-XL, mRFP-TMD VAMP2 or mRFP alone (Fig 6).

Even though Bcl-XL is able to dimerize in absence of its TMD under some conditions, we analyzed how the absence of its TMD

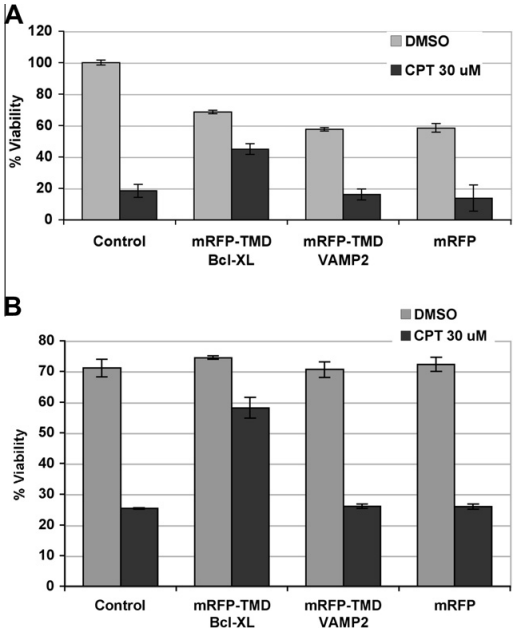

Fig. 5. Protection of mRFP-TMD Bcl-XL against camptothecin-induced cell death. HEK293 cells transfected with myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone were treated with DMSO or with 30 μM camptothecin for 24 h starting 12 h post-transfection. (A) Cells were analyzed using the Cell Proliferation Kit II (XTT) (Roche). Results of two independent assays carried out in triplicates are shown. (B) Cells were analyzed by trypan blue exclusion. Results of three independent assays carried out in triplicates are shown. Error bars indicate standard deviation.

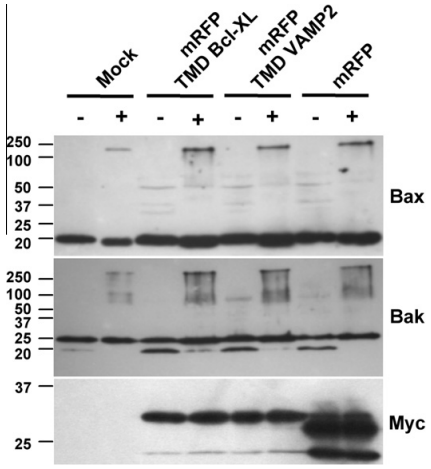

Fig. 6. Effect of overexpression of chimeras in Bax and Bak oligomerization. HEK293 cells were transfected with vectors encoding myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP, treated with 30 μM camptothecin for 24 h and the effect on Bax and Bak oligomerization analyzed by crosslinking with BSOCOES. Fractions from each sample were analyzed by western blot using antibodies against Bax, Bak or myc (to detect overexpressed proteins). Sizes of molecular mass markers run alongside are indicated on the left in kDa. –, without BSOCOES; +, with BSOCOES.

or the presence of a TMD from an unrelated protein affected its dimerization. For this, we constructed a new vector to express Bcl-XL without its TMD, as well as another vector encoding Bcl-XL with its TMD replaced by the second TMD of Pit 2, a plasma membrane sodium transporter [30]. We expressed these constructs in cells and carried out crosslinking assays using BSOCOES. Under these conditions, we could detect dimers of full-length Bcl-XL (Fig 7, lane 2) and no dimers of Bcl-XL lacking its TMD (Fig 7, lane 4). Curiously, Bcl-XL with its TMD replaced by the second TMD of Pit2 was also able to dimerize (Fig 7, lane 6).

Analysis of the sequences of the TMD of Bcl-XL and the second TMD of Pit 2 revealed the presence of the sequence motif GXXXXG (where G is glycine and X, any amino acid), in tandem in Bcl-XL (GMTVAGVVLLG) and alone in the TMD of Pit2 (GSVLLG). Note that five out of six amino acids are conserved between sequence GVVLLG in Bcl-Xl and sequence GSVLLG in the second TMD of Pit2. Since the sequence motif GXXXG had been reported to be involved in dimerization of the BH3-only Bcl-2 family member BNIP3 through its TMD, and also in other proteins [31–38], which is very similar to the motifs found in the TMD of Bcl-XL and the second TMD of Pit2, we constructed a new vector where the TMD of Bcl-XL was replaced by the third TMD of Pit2, which lacks sequence GXXXXG. Crosslinking assays with this new chimera indicated that it was unable to dimerize (Fig 7, lane 8).

We analyzed the subcellular localization of these chimeras, with myc-BclXL localizing to mitochondria as expected and the remaining chimeras showing a diffuse localization in the cell (Fig 4B). This also indicates that sequences involved in dimerization and mitochondrial targeting within the TMD of Bcl-XL are different, since

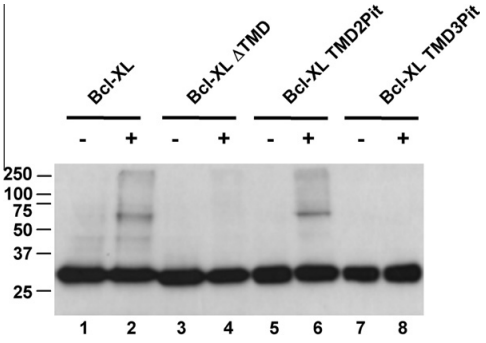

Fig. 7. Effect of different TMDs in Bcl-XL dimerization. Full length Bcl-XL (Bcl-XL), Bcl-XL without its carboxyl terminal TMD (Bcl-XL ΔTMD), Bcl-XL with the second TMD of Pit 2 (Bcl-XL TMD2Pit) or Bcl-XL with the third TMD of Pit 2 (Bcl-XL TMD3Pit) all preceded by a myc tag, were overexpressed in HEK293 cells, treated with the crosslinker BSOCOES and analyzed by western blot with an anti-myc antibody. –, without BSOCOES; +, with BSOCOES.

the second TMD of Pit2 is able to induce oligomerization but not mitochondrial localization.

4. Discussion

We have shown that the transmembrane C-terminal domain of Bcl-XL can directly participate in oligomerization. This is a mode of self-association unreported for Bcl-XL. Bcl-2 family members control their activities through a complex network of protein-protein interactions, posttranslational modifications, transcriptional control or protein degradation [11]. Conformational changes induced upon heteromeric or homomeric protein interactions are very important for their activities.

Bcl-XL has been shown to dimerize by different mechanisms under several conditions [12–17]. Many of these studies, as well

as structural studies, used Bcl-XL mutants lacking the C-terminal TMD under non-physiological assay conditions, although its presence is likely to affect the overall fold of the protein, conformational changes and protein interactions.

Bcl-XL has been reported to regulate apoptosis by heterodimerization-dependent and -independent mechanisms. The former implies binding to pro-apoptotic proteins; the latter, could depend, at least in part, on the formation of an ion channel that can counteract the effects of MOM permeabilization by proteins like Bax or Bak [39]. Interestingly, C-terminal cleavage products of Bcl-XL can form a pore large enough to allow cytochrome c release [40], changing a pro-survival protein into a pro-apoptotic one, what has been also described for Bcl-2 upon binding of a Nur77-derived peptide [41]. Zheng et al. [29] reported changes in mitochondrial morphology and protection against apoptosis mediated by a YFP-TM Bcl-XL fusion protein, suggesting that this TMD could be required for a bioenergetic function of Bcl-XL distinct from BH3 domain sequestration. Our results suggest that oligomerization mediated by the TMD of Bcl-XL could be involved in these activities.

The TM domains of other Bcl-2 family members have been also reported to be involved in protein dimerization, like the TMD of BH3-only protein BNIP3 [37] or the TMD of Bax [42]. In the case of BNIP3, the sequence GXXXG has been shown to directly participate in dimerization. Interestingly, a similar sequence is present in the TMD of Bcl-XL, but in tandem (GMTVAGVVLLG: GMTVAG and GVVLLG), with one more amino acid between the two glycines (GXXXXG). The presence of sequence GXXXG in tandem had been previously described in other proteins [35,38]. Curiously, a very similar sequence containing that motif is also present in the second TMD of Pit2 (GSVLLG), which we used to replace the TMD of BclXL expecting to block dimerization (before we analyzed the TMD sequences), and Bcl-XL with that TMD also dimerizes. BclXL with its TMD replaced by the third TMD of Pit2, which does not contain that sequence motif, does not dimerize, strongly suggesting the importance of motif GXXXXG in dimerization.

Sequence GXXXG was first reported to be involved in TMD dimerization by Engelman and coworkers [34,43]. Furthermore, within these motifs, residues V and L are also usually present close to G residues, as is the case for the TMD of Bcl-XL and the second TMD of Pit2. Sequence specificity in the dimerization of transmembrane alpha-helices was first reported by Lemmon et al. [31], reporting later that the pattern LIxxGVxxGVxxT is involved in dimerization [44]. Furthermore, the GXXXG motif has been involved in the formation of a membrane channel by the *Helicobacter pylori* vacuolating toxin [38], where this motif appears in three tandem repeats. This motif has also been found in several other proteins, like subunits e [45] and g [46] of yeast mitochondrial ATP synthase, APH-1 [47], ABCG2 [48], the SARS coronavirus spike protein (were it is involved in trimerization) [49], prion protein [50], amyloid precursor protein [51], the two-peptide bacteriocin lactococcin G [52], carnitine palmitoyltransferase 1A [53], ErbB [54], protein FliH and its Type III secretion homologue YscL [55], the Japanese encephalitis virus precursor membrane (prM) protein [56], the PufX polypeptide of *Rhodobacter sphaeroides* RC-LH1 photosynthetic complex [57], human organic anion transporter 1 (hOAT1) [58], VDAC1 [59] and the NS4B protein of hepatitis c virus [60]. A review about some of these proteins can be seen in Senes et al. [61].

Although in the TMD of Bcl-XL the two glycines are separated by four residues instead of 3 as in the GXXXG motif, our results strongly suggest that this motif is mediating interaction of Bcl-XL molecules through their TMDs, since the second TMD of Pit2, with 5 out of 6 conserved residues within that GXXXXG motif also allows dimerization of the protein, whereas the third TMD of Pit2, which lacks that motif, does not dimerize when fused to Bcl-XL.

In summary, we have shown that the TMD of Bcl-XL is involved in protein dimerization and that motif GXXXXG is very likely responsible for this interaction. This will most likely have important implications in Bcl-XL function.

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Review

Modulation of apoptosis by early human papillomavirus proteins in cervical cancer

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CD95

ABSTRACT

Cervical cancer (CC) constitutes a major women health problem. Clinical, molecular, and epidemiological investigations have identified persistent infection with high risk human papillomavirus (HR-HPV) as the major cause of CC. HR-HPVs lead to development of cervical carcinoma, predominantly through the action of E5, E6 and E7 viral oncoproteins. After HR-HPV infection, viral proteins employ strategies to modulate apoptosis. The E2 viral protein induces apoptosis in both normal and HPV-transformed cells through activation of caspase-8. The E5 protein can impair CD95L- and TRAIL-mediated apoptosis, which suggests that it may prevent apoptosis at early stages of viral infection. E6 inhibits apoptosis through the proteolytic inactivation of pro-apoptotic proteins such as p53, FADD, or procaspase-8, employing the ubiquitin proteasome pathway, or through interactions with proteins that form the death-inducing signaling complex (DISC) such as TNF-R1. On the other hand, E7 oncoprotein expressing cells are usually predisposed to undergo apoptosis. Useful targets for therapeutic strategies would interfere with expression or function of HR-HPV proteins to eliminate cells that express viral oncoproteins. In this review, we summarize the available data on the interaction of early HPV proteins with cellular factors that promote cell death, and the functional consequences of these interactions on apoptosis.

Contents

1. Introduction

Cervical cancer (CC) and precancerous cervical lesions constitute a major women health problem. Clinical, molecular, and epidemiological investigations have identified high risk human papillomavirus (HR-HPV) as the major cause of CC and cervical dysplasia [1]. Papillomavirus are small DNA viruses that infect various epithelial tissues, replicate in the stratified layers of skin and mucosa, and usually give rise to benign lesions such as warts or papillomas. HPVs can be classified as either HR-HPV or low risk types (LR-HPV) on the basis of their genital clinical associations. The HR-HPV types, such as HPV-16 and 18 are commonly associated with lesions that can progress to high grade intraepithelial neoplasia and ultimately to carcinoma, while the LR-HPV types, such as HPV-6 and 11 are found associated primarily with benign genital lesions, which rarely progress to cancer [2]. HR-HPVs code for at least three proteins with growth-stimulating and transforming properties (E5, E6, and E7). E5 protein contributes to cellular transformation by increasing the mitogenic stimulus from

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growth factor receptors to the nucleus [3]. The E6 and E7 proteins stimulate cellular progression through the G1/S transition despite the presence of various G1 arrest signals in their host cells. The best-described target for E6 is the p53 tumor suppressor protein. Binding of E6 to p53 promotes degradation through an ubiquitin-dependent mechanism [4]. E7 is best known for its interaction with pRb [5]. E2 encodes a regulatory protein, which in the genital HPVs is involved in the negative regulation of the viral promoter that directs expression of the E6 and E7 oncogenes [6,7]. One characteristic of HR-HPV-related carcinogenic progression is the frequent integration of the viral genome into the host chromosomes in a manner that results in the loss of the viral E2 transrepressor protein expression consequently maintaining high E6/E7 expression levels [8]. These observations suggest that the loss of E2 expression may be an important step in HPV-associated carcinogenesis. Also, it was reported that high anti-apoptotic c-FLIP (Caspase-8/FLICE inhibitory protein) expression is present in E2-disrupted cervical lesions, compared with cervical lesions that express E2, suggesting that overexpression of c-FLIP occurs mainly after HPV integration [9]. It is well known that E6 and E7 oncoproteins are necessary for the maintenance of the transformed phenotype [10,11] but their expression alone is not sufficient to transform human cells by a single hit mechanism. Additional alterations are required for the infected cells to be fully transformed [12]. It is important to point out that not all persistent HR-HPV infections lead to cervical carcinogenesis. The existence of intra- and extra-cellular surveillance strategies which prevent the accumulation of malignant cells has been demonstrated experimentally [1]. One mechanism for the elimination of these malignant cells is via apoptosis [13].

2. The apoptotic pathways

Apoptosis is a form of programmed cell death that is regulated physiologically and genetically, and plays a central role in development, morphogenesis, normal cell turnover and immune system function [14,15]. Apoptosis contributes to the elimination of chemotherapy damaged, irradiation damaged or viral infected cells [16,17]. Abnormal apoptosis is involved in various diseases such as autoimmune diseases and cancer [18,19]. Morphological features of apoptosis are well characterized. These include nuclear membrane breakdown, chromatin condensation and fragmentation, cell membrane blebbing, and formation of apoptotic bodies. Two main apoptotic routes have been identified [15]: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. In the extrinsic death receptor pathway, receptors are activated specifically by their cognate ligands. For instance, TRAIL, TNF and FasL have been shown to induce the caspase cascade by binding and activating their membrane receptors, DR4/TRAIL-R1 and DR5/TRAIL-R2, TNF receptor-1 (TNFR1), and Fas (CD95), respectively [20,21]. These receptors belong to the TNF receptor family, in which other related receptors, such as the p75 nerve growth factor receptor, and CD40, are also included [22,23]. The receptor proximal events have been best characterized for Fas, where stimulation of CD95 by its ligand results in the aggregation of the receptor. This is achieved, through its intracellular death domains (DD), leading to the recruitment of two key signaling proteins that together with the receptor form the death-inducing signaling complex (DISC) [24]. Fas associated death domain (FADD) protein couples through its C-terminal DD to CD95 receptors and recruits caspase-8/FADD-like interleukin-1beta-converting enzyme (FLICE) through its N-terminal death effector domain (DED) to the DISC [24,25]. DISC formation results in proteolytic activation of caspase-8/(FLICE), which leads to gradual proteolytic cleavage of caspases and caspase-activated deoxyribonuclease [25,26]. For CD95-mediated apoptosis, two pathways have been shown, which are used in different cell types (types I and II) [27].

Type I cells require activation of caspase-8 at the DISC closely followed by activation of caspase-3 (see Fig. 1). Blocking the release of apoptogenic factors from mitochondria by overexpression of Bcl-2 or Bcl-X_L has no effect on caspase-8 or caspase-3 cleavage or on the CD95 sensitivity of these cells. This suggest that type I cells have developed a way to partially bypass mitochondrial functions, as they activate caspase-8 followed by caspase-3 independent of mitochondrial activity. In type II cells, a low ability of the DISC to process caspase-8 was detected, despite normal expression levels of the DISC components CD95, FADD, and caspase-8 [27]. As a result, these cells require an amplification step initiated by the cleavage of Bid by the small number of activated caspase-8 molecules, followed by translocation of the truncated Bid to the mitochondria where it is responsible for triggering the release of cytochrome *c* [28,29]. Mitochondria in these cells may function as an amplifier for low caspase activity generated at the DISC, activating both caspase-8 and caspase-3. Only in these cells activation of caspases can be blocked by Bcl-2 or Bcl-X_L with strong reduction of apoptosis sensitivity [27,30] (Fig. 1).

A class of virus-encoded apoptosis inhibitory molecules, designated as viral FLICE inhibitory proteins (v-FLIPs), has been described [31]. Also, a cellular homologue of v-FLIPs was identified by different groups and termed c-FLIP [32], FLAME [33], I-FLICE [34]. These molecules are composed of two death effector domains, a structure resembling the N-terminal half of caspase-8. Via DED–DED interaction, v-FLIPs are recruited to the CD95 DISC [31], preventing caspase-8 recruitment and processing and thereby CD95-induced apoptosis.

The intrinsic mitochondrial pathway is used in response to many nonspecific stimuli, e.g., DNA damage, radiation and osmotic stress [15,35], and results in cytochrome *c* release from the mitochondrial intermembrane space to cytosol. In the cytosol, a complex known as the apoptosome is formed from apoptotic protease activating factor-1 (Apaf-1), procaspase-9 and cytochrome *c* [36,37]. Apaf-1 has a caspase recruitment domain (CARD) that allows it to bind to caspase-9, a central P loop motif that binds ATP, and at the COOH terminus WD40 repeats that bind cytochrome *c* [37,38]. Oligomerization of Apaf-1 leads to autoactivation of procaspase-9 that in turn cleaves and activates caspase-3, an event that induces the cleavage of other death substrates [38–40] (Fig. 1).

3. HPV proteins and apoptosis

Upon infection, human pathogenic DNA viruses employ several strategies to modulate the apoptotic response [41]. Loss of expression of pro-apoptotic proteins in many neoplasias might result in defective or delayed apoptosis, thus facilitating tumor growth or survival. For example, it has been reported that HPV-positive cervical carcinomas and cell lines display multiple alterations in the expression profile of initiator and effector caspases [42].

3.1. E2-induced apoptosis in several cell lines

The viral transcription factor E2 plays a major role in both HPV expression and initiation of viral replication. E2 contains an NH2-terminal transcription activation domain and a COOH-terminal DNA binding domain separated by a flexible hinge [43]. It binds as a dimer to 12-bp inverted repeats present in the HPV upstream regulatory region and consequently activates or represses transcription, depending on the distance of E2-binding motifs from the start site [6,44,45]. Repression appears to be mediated by displacing cellular factors from the viral promoter [7]. Interestingly, it was reported that overexpression of the viral E2 protein in HPV-transformed cells represses transcription of E6 and E7 inducing apoptosis and/or growth arrest. For example, it has been reported that BPV or HPV-18 E2 gene transfection in HeLa cells induces p53-independent apoptosis as well as p53-dependent growth arrest [46,47]. Also, E2-induced apoptosis can occur in some cells devoid of HPV DNA, indicating that the pro-apoptotic activity of E2 is independent of other viral functions and of the cell type. The pro-apoptotic property of HPV-18 E2 has been shown

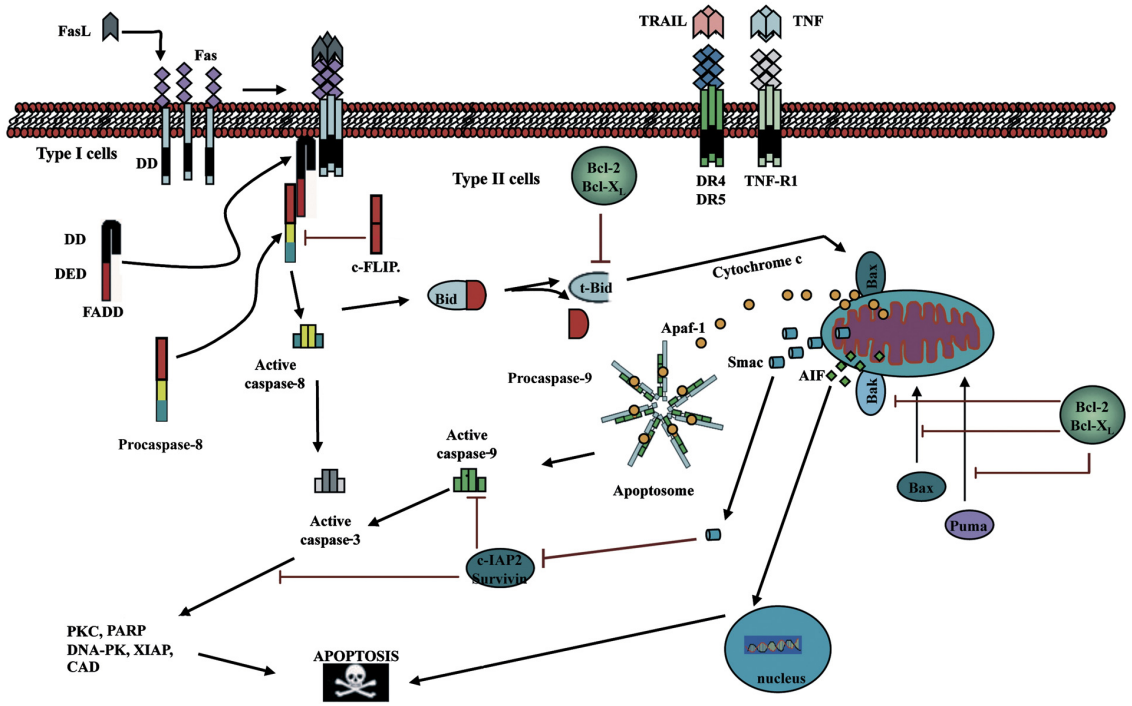

Fig. 1. Apoptosis pathways. In the extrinsic death receptor pathway, FasL binds Fas and induces trimerisation of Fas, which recruits FADD through interaction with death domains (DD). Fas–FADD complex binds procaspase-8 forming a complex named DISC. Activated caspase-8, induces apoptosis through the activation of caspase-3. The activation mechanism of TRAIL receptors DR4, DR5 and TNF-R1 is similar to that of Fas activation. Type I cells require activation of caspase-8 at the DISC followed by caspase-3 activation. In type II cells, caspase-8 cleaves full length Bid, resulting in truncated tBID, a pro-apoptotic protein that activates Bax, resulting in the release of cytochrome *c*, Smac and AIF, from mitochondria. Bcl-2 and Bcl-X_L inhibit t-Bid. c-FLIP is an anti-apoptotic competitor of caspase-8. In the intrinsic mitochondrial pathway, Bax and Puma translocate from the cytosol to the mitochondrial membrane as a result of DNA damage. AIF is the main mediator of caspase-independent apoptosis like programmed cell death, which may contribute to DNA and nuclear fragmentation. Smac is a pro-apoptotic protein that inhibits IAPs (c-IAP2, survivin). Cytochrome *c* is released into the cytosol and binds to Apaf 1 to self-activate caspase-9 (apoptosome). Subsequently, caspase-9 activates downstream caspases [modified according to 27,37,38]. Black boxes represent death domains in FADD and death receptors; red box represent death effector domains in FADD, procaspase-8 and c-FLIP.

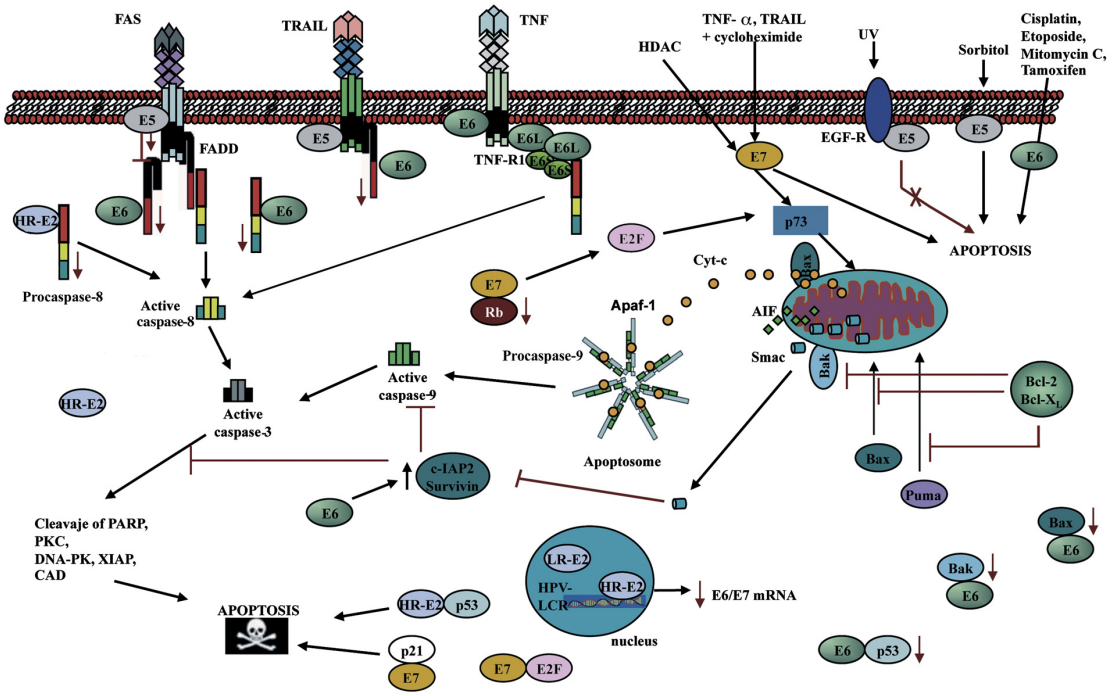

Fig. 2. Modulation of apoptosis by HR-HPV early proteins. The picture shows several interactions of viral oncoproteins with cellular proteins and their effects on apoptosis. E2 protein can induce apoptosis in both HPV-transformed and non-HPV-transformed cell lines through downregulation of E6/E7 mRNA and through binding with p53, respectively. E5 impairs the formation of the death-inducing signaling complex triggered by FasL and TRAIL. Frequently, E6 protein is associated with apoptosis inhibition because it targets pro-apoptotic proteins, such as p53, Bax, and Bak for proteolytic degradation. Besides, E6 protein can protect cells from death receptor-induced apoptosis blocking apoptotic signal transduction and inducing the expression of IAPs. E7 oncoprotein expressing cells are usually predisposed to undergo apoptosis, in part due to degradation of the anti-apoptotic protein pRb and E2F-1 binding. The ↓ represent the degradation of cellular proteins. E6L, E6 large, E6S, E6 small. See the text for more details.

to depend on induction of the extrinsic pathway through activation of caspase-8 and a threshold expression level of the E2 protein [48]. The mechanism of this activation involves the binding of E2 protein directly to the DED of caspase-8 through non-death fold domain interaction, in a FADD independent way. The amino-terminal domain of HPV-18 E2 protein contains a 27 amino-acid α-helix, which is necessary and sufficient to induce caspase oligomerization and cell death [49]. Besides, the N-terminal transactivation domain from HPV-18 E2 protein is sufficient for p53-independent promotion of cell death [48].

In contrast, the HPV-16 E2 protein can induce apoptosis via a p53-dependent pathway in HPV-transformed cells, non-HPV-transformed cells and untransformed normal cells [50,51]. The mechanism for this induction involves the binding of E2 to p53. Interestingly, neither the ability of p53 to regulate transcription nor the ability of p53 to bind DNA, are required for HPV-16 E2-induced apoptosis in non-HPV-transformed cells [51]. This cell death induced by E2 viral protein in both normal and HPV-transformed cells precludes the use of E2 in the treatment of HPV-induced cancer. However, recently it was reported that VP22–E2 fusion protein (E2p53m), in which E2 has been mutated at positions that reduce the binding of E2 to p53, specifically induces apoptosis in HPV-transformed cells while having little effect on normal cells [52]. Furthermore, interaction between p53 and E2 may be important for the HPV life cycle because it was reported that p53 can downregulate HPV-16 DNA replication via E2 protein interaction [53].

Besides, HPV-16 E2 also induced apoptotic cell death through the binding of E2 to the viral promoter in HPV-transformed cells [50,51]. It has been shown that HPV-16 E2 protein induces apoptosis through caspase-8 activation, as previously determined for HPV-18 E2 [49]. Although, E2 protein from HPV-16 induces apoptosis through p53-dependent pathways and E2 protein from HPV-18 induces apoptosis through p53-independent pathways, both proteins activate caspase-8, suggesting that the mechanism of apoptotic cell death was probably conserved between the E2 proteins from these two viruses.

There is also some evidence suggesting that the expression of the HPV-31 E2 protein in HPV-negative normal human foreskin keratinocytes (NHK cells) might induce apoptotic cell death [54]. In contrast, E2 proteins from LR-HPVs types 6 and 11, which are associated with benign lesions, do not cause cell death in HeLa cells. Subcellular localization of the E2 proteins appeared strikingly different in high and low risk HPVs. The E2 proteins from low risk viruses are exclusively nuclear, whereas proteins from HR-HPVs are located in both the nucleus and the cytoplasm. Thus, cytoplasmic localization of HR-E2 proteins correlates with induction of apoptosis through caspase-8 activation. In contrast, E2 proteins from LR-HPVs do not induce apoptosis probably due to their exclusive nuclear localization [55]. The E2-induced apoptosis is shown in Fig. 2.

3.2. Inhibition of apoptosis by E5

At early stages of HPV-16 infection, when the viral genome is episomal, E5 mRNA is one of the most abundant viral transcripts [56]. It has been proposed that inhibition of death receptor-mediated apoptosis in human keratinocytes, needed to prevent apoptosis at early stages of viral infection, is a primary function of the HR-HPV E5 protein. It was reported that HPV-16 E5 protein impairs CD95L- and TRAIL-mediated apoptosis in HaCaT cells by: (a) downregulating the total amount of CD95 receptor and reducing CD95 surface location; and (b) altering the formation of the DISC triggered by TRAIL [57].

Raft cultures of E5-expressing keratinocytes were completely protected from FasL- or TRAIL-induced cell death [58]. Likewise, when UV radiation is used to induce stress, E5-expressing human keratinocytes are protected from apoptosis [59].

In contrast, the HPV-16 E5 protein sensitizes human keratinocytes to apoptosis induced by osmotic stress, perhaps due to cell membrane modifications caused by this strongly hydrophobic molecule [60]. The modulation of apoptosis by this and other HR-HPV oncoproteins is summarized in Fig. 2.

3.3. The role of E6 in apoptosis

One of the functions of the HR-HPV E6 oncoproteins is the proteolytic inactivation of certain pro-apoptotic proteins such as p53 [4], Bak [61], FADD [62], procaspase-8 [63], or c-Myc [64] through the ubiquitin proteasome pathway, which obviously affects apoptosis. For example, Aguilar-Lemarroy et al., found that E6 and E6/E7 immortalized keratinocytes were resistant to CD95 ligand while E7 immortalized keratinocytes are sensitive. Only E6 immortalized keratinocytes were sensitized to ligand-induced cell death by inhibition of proteosomal degradation, followed by re-expression of p53 and c-Myc but not of other pro-apoptotic proteins such as Bax. In this work, the elevation of p53 was not sufficient to cause apoptosis in E6/E7 or E6 cells when incubated with a proteasome inhibitor, unless CD95 ligand was provided [65], suggesting that in E6 immortalized keratinocytes the downregulation of p53 constitutes an important event to inhibit CD95 related apoptosis. The interaction between p53-dependent apoptosis and CD95 receptor/ligand system has been shown; for example, CD95 receptor is upregulated by p53 in response to DNA damage by anticancer drugs [66,67]. Besides, p53 activation transiently increased surface CD95 expression facilitating the transport from the Golgi complex [68]. It is also possible that a p53-independent mechanism could be partially responsible of CD95-induced apoptosis.

In addition, it has been reported that malignant HPV-16 positive cervical carcinoma cells that were insensitive to either CD95 or TNF-α ligands do not form a functional DISC [69]. It is possible that some viral proteins interact with the death receptors and protect cells from TNF or FasL-induced apoptosis. For example, it has been reported that E6 can protect cells of different species (mouse and human) and tissues (fibroblast, LM cells; osteosarcoma, U2OS; and histiocyte/monocyte, U937) from TNF-mediated cell death. E6 protects both LM and U2OS cells from TNF-mediated cell death in a p53-independent manner. The inhibition of TNF pathway in the presence of E6 can be related to the inability of the TNF-R1 intracellular death domain to interact with TRADD, because E6 binds to the C-terminal part of TNF-R1 [70], blocking apoptotic signal transduction. It is also possible that E6 inhibits TNF-mediated apoptosis through induction of cellular inhibitor of apoptosis protein 2 (cIAP-2) [71], or through the overexpression of Bcl-2 and the downregulation of Bak [72], in the above mentioned cells. Given that HPV-16 E6 transactivates survivin promoter activity, it can be speculated that *survivin* gene also plays an important role in the anti-apoptotic function mediated by E6 [73] (Fig. 2).

E6 was also shown to protect U2OS cells from Fas-induced apoptosis, through binding and degradation of FADD, which prevents caspase-8 and caspase-3 activation. However, E6 does not affect apoptotic signaling in U2OS cells through the mitochondrial pathway [62], suggesting that E6 has a more generalized effect on signaling by death ligands. Furthermore, it was observed the accelerated degradation of FADD and procaspase-8 in some cell lines expressing HPV-16 E6, which impairs TRAIL-mediated apoptosis [63]. The large and small isoforms of HPV-16 E6 bind to procaspase-8, but only the large isoform accelerates the degradation of this enzyme [74]. However, the expression of E6 does not always result in protection from TNF family member-mediated apoptosis. It was reported that U2OS cells expressing low levels of E6 are protected from TNF-mediated apoptosis, while high levels of this oncogenic protein sensitize these cells [75]. Recently, it was demonstrated that when the large and small isoforms of HPV-16 E6 are expressed together they can sensitize cells to TNF and that this sensitization can be achieved only at high levels of E6 expression. With this increased level of large and small isoforms of E6 comes the ability to form the E6 complexes that are capable of binding

Table 1
Modulation of apoptosis by E6.

Model	Conditions	Reference
E6 sensitizes apoptosis		
Early passage HME cells	P53 (−), tamoxifen, HPV-16 E6	[85]
Primary HKCs	Wt P53, caspase-3, ↓ p21, cisplatin, etoposide, MMC, HPV-16 E6	[86]
Spontaneously immortalized human foreskin keratinocytes (NIKS cells)	Apoptosis involving caspase activation and the mitochondrial pathway to DNA damaging and chemotherapeutic agents, activation of Cdc2, HPV-16 E6	[87]
Mouse fibroblast cell line J2-3T3	Wt P53 expression, atractyloside, participation of ICE-like protease sensitive mechanism, HPV-16 E6	[89]
HPV-negative cervical cancer cell line C33-A	MMC, SSP, gamma irradiation, HPV-18 E6, P53- and Rb-independent pathway	[90]
Human ovarian cancer cell line A2780 and human colon cancer cell line HCT-116	Apoptosis induced by TNF, NF-κB inhibition, release of cytochrome *c*, HPV-16 E6	[91]
E6 inhibits apoptosis		
Human kidney cell line 293	Inhibition of Bak-induced apoptosis, ↓ Bak, E6 proteins from high and low risk HPV types	[61,84]
Human osteosarcoma cells U2OS	Interaction of E6 with FADD, inhibition of both caspase-3 and caspase-8, inhibition of FAS-mediated apoptosis, HPV-16 E6	[62]
Human colon cancer cells HCT116 and human osteosarcoma cells U2OS	↓ FADD, ↓ procaspase-8, inhibition of TRAIL-mediated apoptosis, presence of the large isoform HPV-16 E6	[63,150]
Primary HKCs	CD95-mediated apoptosis, ↓ Myc, ↓ P53, HPV-16 E6	[65]
Human osteosarcoma cells U2OS, mouse fibroblast LM cells, and myeloid leukemia cell line U937	Interaction of E6 with TNF-R1, inhibition of TNF-mediated apoptosis, HPV-16 E6	[70]
Human oral keratinocytes	↑ NF-κB, ↑ cIAP-2, ↓ TNF-mediated apoptosis, HPV-16 E6	[71]
Human laryngeal cancer cell lines	↓ Bak, ↑ Bcl-2, HPV-16 E6	[72]
Primary epidermal keratinocytes	Inhibition of UVB-induced apoptosis, prevented release of AIF, cytochrome *c* and Omi from mitochondria; inhibition of Bax activity by E6 protein	[77]
Lens of transgenic mice	Mouse lens development, P53-independent mechanisms, HPV-16 E6	[78]
Primary HKCs	E6 inhibits apoptosis induced during serum-calcium differentiation, prolonged expression of Bcl-2 and significantly reduced elevation of Bax, levels downregulation of P53, HPV-16 E6	[79]
Human osteosarcoma cells U2OS	Bax-dependent apoptosis, downregulation of Bax mRNA expression and protein stability by HPV-16 E6 protein	[80]
Cervical cancer cells	↓ E6 leads to ↑ P53, ↑ Puma, activation and translocation of Bax to the mitochondrial membrane, cytochrome *c* release, and activation of caspase-3	[81]
HaCaT cells	UVC, MMC, serum starvation, mutations in the P53 loci, HPV-16 E6	[82,83]

Abbreviations: MMC, mitomycin C; SSP, staurosporine; Wt, wild type; HME cells, human mammary epithelial cells; HKCs, human keratinocytes; TNF-R1, tumor necrosis factor receptor 1; GADD34, growth arrest and DNA damage protein 34; eIF2, eukaryotic initiation factor 2; FADD, fas-associated death domain; cIAP-2, inhibitor of apoptosis 2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; HR-HPV, high risk human papillomavirus; AIF, apoptosis-inducing factor.

to TNF R1 and procaspase-8 at the same time. As a result of this pseudo-DISC formation, cells become sensitive to TNF [76]. In contrast, high levels of E6 inhibit Fas-mediated apoptosis in U2OS cells through E6 large [75,76]. In conclusion, both the levels and the ratio between E6 large and E6 small are important in modulating TNF- and Fas-mediated apoptosis. It is necessary to perform studies on the in vivo E6 protein expression levels regarding both apoptosis and HPV life cycle.

Although E6 does not interfere with the mitochondrial apoptotic pathway in both U2OS and HCT116 cells [62,63] it has been shown that E6 protein prevented release of apoptotic factors such as AIF, cytochrome *c* and Omi from mitochondria of UV-damaged primary epidermal keratinocytes and preserved mitochondrial integrity [77]. Besides, this viral oncoprotein inhibits apoptotic events necessary for the normal development of the lens in E6 transgenic mice [78]. In contrast with a previous report [65], it has been shown that the inhibition of Bax activity is crucial for the anti-apoptotic function of the E6 oncoprotein [79–81]. For example, it was reported that E6 inhibited apoptosis during serum and calcium-induced differentiation of human keratinocytes; E6 expression and p53 degradation correlate with reduced levels of both Bax mRNA and protein, and increased expression of Bcl-2 [79,80]. Consistent with these reports, it was demonstrated that the inhibition of Bax plays a main role in the anti-apoptotic function of the viral E6 oncogene in cervical cancer cells in a p53 and puma-dependent manner, because inhibition of E6 expression in cervical cancer cells leads to the activation and translocation of Bax to the mitochondrial membrane [81]. It has been shown that E6 inhibited apoptosis caused by UVC, mitomycin C, and serum starvation in immortalized human keratinocytes (HaCaT) bearing mutated alleles of p53 [82,83], suggesting that p53 is not involved in this mechanism. In addition, E6 inhibits Bak-mediated apoptosis in 293 cells through the degradation of Bak protein by HR and LR-HPV E6 proteins [61,84]. However, LR-HPV E6 is less effective than the HR-HPV E6 proteins in the inhibition of Bak-mediated apoptosis, indicating that the ability of HPV to circumvent Bak-induced apoptosis may contribute to the oncogenic potential of the virus [84]. Therefore, it is probable that in some circumstances E6 may inhibit the mitochondrial pathway through inhibition of pro-apoptotic members of the Bcl-2 protein family. To date, only some cellular proteins have been described to play a role in the mechanism through which E6 inhibits cell death, but the picture is still incomplete.

Depending on the cell type, the viral type and the stimulus, the E6 oncoprotein from HR-HPVs can also activate apoptosis. For example, early passage p53-defective immortalized human mammary epithelial cells (HMECs), can be sensitized to apoptosis by HPV-16 E6 in the presence of tamoxifen [85]. Likewise, HPV-16 E6 enhanced the sensitivity of human keratinocytes to apoptosis induced by chemotherapeutic agents [86]. It is possible that in this cellular model E6 contributes to apoptosis through Cdc2 in response to DNA damaging and chemotherapeutic agents [87]. Interestingly, the expression of HPV-16 E6 sensitized murine fibrosarcoma L929 cells to TNF-induced necrosis instead of apoptosis. Cytolysis induced by HPV-16 E6 was independent of p53 degradation and correlated with an increase in the level of reactive oxygen species [88]. HPV-16 E6 expression sensitizes mouse fibroblasts cell line J2-3T3 to mitochondrial membrane permeability transition (MPT)-induced apoptosis in a p53-dependent fashion [89]. Similarly, it was reported that HPV-18 E6 enhanced apoptosis in response to irradiation or chemotherapeutic agents in the HPV-negative (p53 negative) cervical cancer cell line C33-A [90]. HPV-16 E6 sensitizes TNF-induced cytotoxicity of both human ovarian

cancer cell line A2780 and human colon cancer cell line HCT-116. This pro-apoptotic effect of E6 is not p53-dependent, but it is essentially mediated through inhibition of NF-kappaB activation and release of cytochrome *c* [91]. All these reports suggest that E6 can modulate apoptosis through both p53-dependent and independent mechanisms. An overview of apoptosis modulation by E6 described to date is provided in Table 1.

3.4. E7 and apoptosis induction

The multiple functions of E7 oncoproteins from HR-HPVs are sufficient to immortalize primary human keratinocytes [92,93]. These oncoproteins inhibit differentiation [93,94] and activate cell cycle progression, mainly due to the disruption of the pRb-E2F complex, releasing active E2F and trans-activating several genes involved in DNA synthesis [93]. In addition, E7 is a potent inhibitor of $p21^{CIP1}$ and $p27^{KIP1}$ activity, avoiding normal G1 checkpoint control [94,95]. The E7 oncoprotein of HPV-16 induces anti-apoptotic pRb degradation through the ubiquitin proteasome pathway [96,97], suggesting that E7 might support apoptosis. It was reported that cells that express the HPV-16 E7 oncoprotein are predisposed to undergo apoptosis [78,98]. For example, HPV-16 E7 induces apoptosis in the retina from transgenic mice expressing this viral oncogene. P53 is required for the induction of apoptosis in this model, because mice expressing E7 in a p53 nullizygous background develop retinal tumors instead of undergoing retinal degeneration [98]. Similarly, HPV-16 E7 induced both p53-dependent and independent apoptosis when expressed in the lens of transgenic mice. Interestingly, the E6 transgene reduces levels of apoptosis induced by E7 in neonatal lenses [78].

In cell line models, several reports suggest also the induction of apoptosis by the E7 protein. For example, E7 activates apoptosis in immortalized rodent fibroblasts (NIH3T3 cells) deprived of serum survival factors, through both conserved region 1 (CR1) and CR2 of the E7 oncoprotein. In this model, the ability to induce apoptosis is a common property of the E7 proteins belonging to both benign (HPV-1, 6) and malignant HPV types (HPV-16) [99]. In addition, it was reported that simultaneous expression of E7 and p21 proteins induces cell death in U2OS cells, possibly because of conflicting growth control signals. E7/p21-induced cell death is associated with the activation of a mediator of apoptosis, namely cathepsin B [100]. In primary human keratinocytes, the HPV-16 E7 protein increased both spontaneous and TNF-α-induced apoptosis. Furthermore, co-expression of E6 abrogated E7-mediated apoptosis by TNF [101]. Both TNF-α and TRAIL induced apoptosis in E7 expressing human keratinocytes when administered in combination with the protein synthesis inhibitor cycloheximide, but the apoptotic response to TRAIL was significantly more rapid and efficient compared with the response seen after TNF-α treatment. This is not due to upregulation of expression of TNF or TRAIL receptors by E7, because receptors are present in equal amounts [102]. It is possible that E7-induced apoptosis is related with the interaction between E7 and E2F1. This complex could activate E2F1 driven transcription which contributes to increased apoptosis [103]. Besides, in cytokine-treated NHKs, free E2F-1 may further enhance cell death by directly inhibiting anti-apoptotic signaling from TNF receptor-associated factor 2 (TRAF2) at the level of the receptor [104]. Cycloheximide blocks de novo synthesis of short lived proteins (such as c-FLIP), and sensitizes cells to ligand-induced apoptosis [105] (Fig. 2).

However, depending on the cell type and the viral type, the E7 oncoprotein from HPVs can also inhibit apoptosis and cause decreased sensitivity to cytokine-mediated cell death. For example, it was reported that the HPV-16 E7 oncoprotein inhibits TNFα-mediated apoptosis and caspase-8 activation in normal human fibroblasts [106]. Similarly, reduced apoptosis in HaCaT cells expressing E7 was also observed after exposition to genotoxic stress, such as the alkylating agent mitomycin C or UVC [82]. The mechanism by which E7 inhibits apoptosis to genotoxic stress in HaCaT cells is not well defined, but it was recently reported that HPV-16 E7 interacts with the pro-apoptotic cellular factor Siva-1 and inhibits apoptosis in UV radiation-exposed HaCaT cells. Interestingly, HPV-16 E7 appeared capable to interfere in vitro with the binding of Siva-1 to Bcl-X_L, and it is possible that released Bcl-X_L could fully exert its anti-apoptotic function without the negative interference generated by the physical interaction with Siva-1 [107]. Besides, E7-expressing HaCaT cells modulated expression of several genes in response to oxidative stress; in these cells, an increased expression was shown of catalase and Bcl-X_L, and a decreased expression of IL-18, Fas, as well as Bad, resulting in the resistance to oxidative stress-induced cell death [108]. These studies further indicate that cell type as well as transformation state determines the sensitivity in response to specific death stimulus.

Interestingly, histone deacetylase (HDAC) inhibition can induce growth arrest and subsequently strong apoptosis in E7 expressing cells. Chromatin acetylation and activation of tumor suppressor genes (for example p73), might be playing an important role in this phenomenon [109,110]. It is possible that a key regulatory event in E7 expressing cells is the degradation of the anti-apoptotic protein pRb [96,97] and the unscheduled intracellular accumulation of E2F-1 which stimulates apoptosis [111]. Overexpression of E2F-1 induces p53-dependent or independent apoptosis. The p53-dependent mechanism involves transactivation of the ARF protein [112,113]. E2F-1 can stabilize p53 via the induction of the p19ARF protein (p14 in humans), which functions by binding directly to Mdm-2 and preventing p53 degradation [113,114]. Additionally, deregulation of E2F-1 either by ectopic expression of E2F-1 or inactivation of pRb family members by HR-HPV E7, promotes apoptosis through activation of Atm kinase and induction of Chk2 expression, leading to increased phosphorylation and activation of p53 [115]. In the p53-independent mechanism, E2F-1 activates p73 transcription leading to the activation of puma, [116], and bax [117] and to an increase in apoptosis [118]. In conclusion, as opposed to E6 oncoprotein it is evident that, in general, E7 favors p53-dependent or independent mechanisms promoting apoptosis.

3.5. Induction of cell death and growth control of cervical cancer cells

Because of the strong relationship between the expression of HR-HPV oncogenes and cervical carcinogenesis, many approaches have been directed against viral oncogenes, including treatment of HPV-positive cervical cancer cell lines with E6 siRNA [119], with E7 siRNA [120], with both E6 and E7 siRNA [121], expression of antisense RNA to E6 and E7 genes either alone or together [122–125], treatment with peptide aptamers targeting the viral E6 or E7 oncoproteins [126,127], induction and activation of p53 by chemotherapy [128], small molecules [129], intrabodies [130], overexpression of p73β [131,132], the combined use of proteasome inhibitors with inductors of cell death (TRAIL, FasL, TNF) [65,133] and overexpression of E2 viral protein to promote apoptosis [52,134]. Experimentally, all these works have in common the decrease in the levels of mRNA or protein encoding E6 and E7 and as a consequence the restoration of p53 function, hypophosphorylation of pRB and finally, growth arrest and apoptosis induction in HPV-positive cancer cells. Thus, blocking viral oncoprotein expression might be an excellent therapeutic strategy against anogenital cancer.

4. Conclusions

Many viruses have developed strategies to block apoptosis, to preserve the cellular machinery necessary for viral gene expression, as well as replication. The virus avoids host-mediated apoptosis through the downregulation of TNF-R1 [135], the inhibition of DISC formation [65,136], and a sustained expression of inhibitors of apoptosis such as c-FLIP [137]. HR-HPVs, have developed mechanisms to evade host

defenses such as apoptosis and immune defense to establish a successful infection [42,138,139]. For example, E5 protein has been shown to protect keratinocytes from both Fas- and TRAIL-mediated apoptosis, through decreasing the cell surface expression of the Fas receptor and inhibiting the formation of the TRAIL DISC [57]. Due to the integration of HR-HPV genome during malignant progression, the E5 gene is not expressed in cervical tumors but large amounts of E5 mRNA and protein have been detected in anogenital low-grade intraepithelial neoplasia [56,140], supporting the possibility that E5 plays a role in early steps of HPV infection to protect infected cells from apoptosis. Similarly, it has been reported that E6 can inhibit apoptosis induced by TNF, FAS and TRAIL, UVB and other apoptotic stimuli [62,63,70,77,82,141]. This suggests that E6 may function to protect cells from apoptosis triggered by a variety of ligands and may therefore play an important role in the persistence and the oncogenic potential of the virus. The fact that E6 protein performs either protection or sensitization to apoptosis suggests that the response will depend on cell type-specific differences, as well as on the cellular environment, cell cycle and the nature of the apoptotic signal. Besides, it is possible that during the viral cycle both inhibition and induction of apoptosis to the same stimulus are required and modulated by E6 levels.

In support to this, recently it was described that HR-HPV proteins (E6 and E7) can activate capases-3, -7, and -9 upon differentiation to induce viral genome amplification. HPV-mediated caspase activation coincides with increased levels of anti-apoptotic factors, such as survivin and Bcl-2, which may be important for maintaining the viability of HPV-positive cells upon differentiation. It is probable that some degree of E6-induced apoptosis occurs during productive replication to make possible postassembly events [142].

Likewise, it has been shown that E7 protein either inhibits or sensitizes cells to apoptosis, but, most studies suggest that E7 have a pro-apoptotic role. The role of E7 in apoptosis induction is inhibited in E6-expressing cells. It is possible that during the viral cycle E6 inhibits the E7-induced apoptosis. Besides, it was reported that HR-HPV E2 protein also induces apoptosis. It is possible that viral genome integration provides a means to avoid E2-induced apoptosis and allow initiation of carcinogenesis through the activities of E6 and E7. To date little is known about the significance of E2-induced apoptosis in the viral life cycle, as well as the effect of E2 in apoptosis when E5 is present in early stages of viral infection. One possibility is that E5 protects infected cells from host-mediated apoptosis in early stages of viral infection and that E2 favours the release of viral particles to escape from cell-induced apoptosis. The induction of apoptosis by other viral proteins in infected cells to allow the virus multiplication has been reported, suggesting that the pro-apoptotic functions play a universal role during the viral cycle [143–145]. It is well known that E6 and E7 proteins expression are necessary for the maintenance of the malignant state; however, it is also possible that E2 could be the first step in the cellular transformation. It was reported that only HR but not LR-HPV E2 can induce genomic instability through interaction with both Cdc20 and Cdh1, activators of the Anaphase Promoting Complex (APC) [146]. It is evident that more work is necessary to explore this hypothesis.

One question between LR- and HR-HPV proteins about modulation of apoptosis is why there are differences between the oncogenic and nononcogenic HPV types in their abilities to modulate apoptosis. Several reports suggest that the cellular localization plays a determinant role. For example, the E2 proteins from low risk viruses are exclusively nuclear and the proteins from HR-HPVs are located in both the nucleus and the cytoplasm. It has been shown that HR-HPV E2 mediates apoptosis induction is through caspase-8 activation, whereas the E2 proteins from LR-HPVs do not induce apoptosis [55]. Likely, HPV-18 E6 protein was found to be located in the nucleus [147] to difference HPV-11 E6 protein that was predominantly expressed in the cytoplasm [148]. Another difference will be the strong interaction between viral proteins with cellular proteins. This hypothesis is supported by several reports. It has been demonstrated that HR-HPV E6 proteins have a stronger effect upon p53 than do the LR-HPV E6 proteins [4,149]. In the same way, HPV-11 E6 protein induce the degradation of the Bak protein to a lesser degree that HR-HPV E6 protein, and the efficiency of the E6 protein in stimulating degradation appears to correlate with its effectiveness in reducing the apoptosis induced by Bak [84]. Besides, it was reported that p53 binds more tightly to the HR-HPV16 E2 protein than it does to the E2 proteins from LR-HPV6 or LR-HPV11.

For many years apoptosis research has focused on caspases and their putative role as sole executioners of programmed cell death. Accumulating information now suggests that lysosomal cathepsins are also pivotally involved in this process [150]. In particular, the role of lysosomal enzymes in initiation and execution of the apoptotic program has become clear in several models, to the point that the existence of a lysosomal pathway of apoptosis is now generally accepted. This pathway of apoptosis can be activated by death receptors, lipid mediators, and photodamage [151]. Now, it is well known that lysosomal enzymes contribute to induction of autophagy. In a recent report, it was described that autophagy can be activated in a cancer cervical cell line in response to a chemotherapeutic agent [152]. As compared with apoptosis, the effect of HR-HPV early proteins on autophagy is poorly understood. Finally, different studies are necessary to understand possible networks among E2, E5, E6 and E7 in apoptosis regulation during natural infection and whether these molecular pathways could be the target of novel therapies for the prevention and treatment of cervical cancer.

Competing interests

The authors declare that they have no competing interests.

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