



**UNIVERSIDAD NACIONAL AUTÓNOMA
DE MÉXICO**

**Instituto de Neurobiología
Campus UNAM-Juriquilla, Qro.**

**ANÁLISIS DE LA PARTICIPACIÓN DE
PROLACTINA EN INTERACCIONES
ENDOTELIO-LINFOCITOS**

T E S I S
**QUE PARA OBTENER EL GRADO DE
DOCTOR EN CIENCIAS BIOMÉDICAS**

P R E S E N T A
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Juriquilla, Querétaro.

Mayo 2005



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Este trabajo se realizó en el Instituto de Neurobiología de la UNAM, bajo la asesoría de la Dra. Carmen Clapp y contó con el apoyo del Consejo Nacional de Ciencia y Tecnología (CONACyT) y de la Dirección de Estudios de Posgrado (DGEP) de la UNAM.

“Le savant n’étudie pas la nature parce que cela est utile; il l’étudie parce qu’il y prend plaisir et il y prend plaisir parce qu’elle est belle. Si la nature n’était pas belle, elle ne vaudrait pas la peine d’être connue, la vie ne vaudrait pas la peine d’être vécue.”

Henri Poincaré
Science et Méthode

”...Bajo un sistema de educación más realista y menos exclusivamente verbal que el nuestro, todo Ángel —en el sentido que Blake da a la palabra— tendría autorización para un banquete sabático, sería inducido y hasta, en caso necesario, obligado a hacer de cuando en cuando, por medio de alguna Puerta Química del Muro, un viaje al mundo de la experiencia trascendental. Si esto lo aterrara, sería una desdicha, sin duda, pero probablemente saludable. Si le provocará una iluminación breve, pero sin tiempo, tanto mejor. En cualquiera de los casos, el Ángel perdería algo de la confiada insolencia que brota del razonamiento sistemático y de la conciencia de haber leído todos los libros.”

“Blake escribió con mucha amargura:
“Siempre he advertido que los Ángeles tienen la vanidad de hablar de sí mismos como de los únicos sabios. Hacen esto con una confiada insolencia que brota del razonamiento sistemático.”

Aldous Huxley
Las Puertas de la Percepción

“Yo sólo sé... que no he cenado.”

“Cenón”

Refrán de la picardía popular mexicana.

DEDICATORIA

A la Universidad Nacional Autónoma de México.

A mis padres Patricia y Horacio.

A mis hermanos Horacio y Marco.

A México, pero no ese en el que se amparan tecnócratas, empresarios y políticos, si no al enorme grupo de gente que día a día resuelve como puede sus necesidades elementales, a pesar de los abusos y miopía de los grupos de poder.

AGRADECIMIENTOS

A mi mamá, maestra, y hasta hoy y después de todo, confidente... Patricia.

A mi papá y maestro, quien paradójicamente hasta hace poco, me enseñó que las preguntas terminan donde inician los prejuicios y la tranquilidad... Horacio.

A mis cómplices, amigos, ejemplos... hermanos... Horacio y Marco.

A Alejandra Ochoa, excompañera de viaje.

A Lupita y Arturo, no sólo por TODA la ayuda, si no además por las experiencias y conocimientos compartidos.

A UNAM y sus instituciones en donde me he formado (o deformado?) no sólo académicamente durante los últimos 18 años: CCHsur, Facultad de Ciencias, IIB e INB.

A las familias Montes de Oca y Balderas porque de ahí soy y porque siempre hay un momento para reír, bromear, compartir y aprender, a pesar de las diferentes cosmovisiones.

A la Dra. Carmen Clapp por lo que me ha enseñado sobre ciencia, por la oportunidad de desarrollar este proyecto en su laboratorio y por el desafío a mi entusiasmo por la ciencia.

A Libia quien me dio oportunidad de iniciar en este fabuloso oficio.

A mis maestros.

A todos los donadores de sangre y pinchadores quienes con su ayuda hicieron posible este trabajo.

A la sección de Tocología del Hospital General Regional no. 1 de Querétaro, por su valiosa ayuda para la obtención de cordones.

A los miembros de mis comités tutorales de maestría y doctorado: los Drs. Ed Heimer, Rogelio Arellano, Alfredo Varela y Carlos Rosales, por sus valiosas preguntas y ayuda en el desarrollo de este proyecto.

A los miembros del jurado Drs. Manuel Aguilar, Maria Eugenia Gonsebatt, Edmundo Lamoyi, Rafael Rubio, Alfredo Varela y Libia Vega por sus sugerencias y comentarios.

A Fernando López Barrera, Gabriel Nava, Daniel Mondragón y Antonio Prado por su asistencia técnica y colaboración.

A mis compañeros del lab: Alejandra C, Ana, Bibi, Carlitos, Ceci, Celina, Claudia, Daniel (Peluzza), Fernando, Francisco, Gabriel, Gaby, Gino (Chino), Javier, Jessica, Jorge, Jorge V., Luís, Luz, Marco (mesié apual), Marcos, Mari Carmen, Mike, Miriam,

Mónica, Salvador, Toño (Ron Toño), Yazmín y Zulma, por las experiencias, tiempo y espacio compartido.

A Leonor Casanova, Pilar Galarza, Lourdes Lara, Martín García, Carmen Vásquez, Elsa Ruiz, Ignacio Caballero, Rafael Silva, Adolfo Dingler, Fernando De Pedro y Dorothy Pless, por su excelente trabajo y ayuda siempre dispuesta.

A los académicos (o ex) del INB: Alfredo Varela, Jesús Colunga, Rogelio Arellano, Alfonso Carabez, Carmen González, Thalia Harmony, Raúl Paredes, Antonio Fernández, Mauricio Díaz, Aurea Orozco, Teresa Morales, Maricela Luna, Carlos Valverde, Jorge Larriva, Gonzalo, Tana, Fernando Barrios, Magda Giordano, Ed Heimer, Jose Luis Díaz, Flavio Mena, Juan Riesgo, Lourdes Valdivia, Manuel Salas, Leopoldo González, Carmelita Salas, Miguel Rodríguez, Maritza Gaxiola, Gabriel Gutiérrez y Francisca Sandoval por su apoyo, ayuda y/o enseñanzas.

A Margarita, en particular por su gran corazón.

A Silvia, especialmente por su coraje.

A Oqui, Ricardo, Fernando, Adrián, Toño, Clelia, Derek, Héctor y Chefer, aunque a veces no cerca siempre entrañables.

A todas las personas del INB y de Querétaro con quienes al convivir cotidianamente la vida fue y es más fácil, divertida e interesante: Nefalí, Fabián, Icnelia, Juan, Rita, Wendy, Verónica, Chelino, Juan Manuel, Estuardo, Toño, Gloria, Bertha F, Ale, Yvonne, Jeans, Juan Pablo, Luís y fam., Felipe, Gerardo, Rafael, Oscar, Ray, Carmen, Sheyla, Ariel, Daniel, Raúl, Nico, Juan (palencia), el Arquí Castillo, Carmen F, Tere, Adriana, Sancho, Marigli (y sus hijos), Lucia, Alejandro, Robert, Vinicio, Coco, Paloma, el Pez, Daniel, Fernando (y a todo el equipo de VB), Rodolfo, Mauricio, Barbara, Carlitos, Adriana, Mane, Bertha, Alejandria e hijas, Jonas, Pollo, Marco y fam., Ricardo y fam., Arturo, Ulises, Nacho, Sandra, Verónica, Juan caracoles, Kalina, Ricardo, David, Moisés, Edson, Gonzalo, Gustavo, Sergio, Felipe, Josefina, Jakelin, Lucia DSG, Ricardo GM, Rocío, el Atlas de Juriquilla, José Luis, Javier, Ricardo, Jorge (el trabas), Oscar, Efrén, los Pumas del CNB, el FBC de La Norma Iztapalacra, Sofia, More, de Pedro, Felipe MO, Memo, Héctor el güero, Hilda, Javier, Paco, Morales, Gabriel H, Carla y Chino, Víctor, Armande, Ricardo, Poncho, Esteban, Abel, el Barney y fam, Manuel, Tabla, de Pedro, More, Adolfo D, etc. etc. Una disculpa a aquellos que se me pasan, pero mi espacio en C: lo hace imposible. Como sea sabemos con quien compartimos sonrisas, preguntas, creencias o unas frias!

A los subestimados y satanizados psicotrópicos, que contienen tanta información a manera de experiencias en dimensiones donde nuestros conceptos, símbolos, miedos, percepciones y creencias pueden ser cuestionados, desnudados, desafiados y vueltos intrascendentes.

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LISTA DE ABREVIACIONES EMPLEADAS

Ab	Anticuerpo
BCR	Bromocriptina
CAM	Molécula de adhesión
CE	Célula endotelial
D-PBS	Dulbecco-PBS
ECM	Matriz extracelular
AF	Adhesiones focales
FAK	Cinasa de las adhesiones focales
FN	Fibronectina
GH	Hormona de crecimiento
GX	Genisteína
HUVEC	Células endoteliales de vena umbilical humana
ICAM	Molécula de adhesión intercelular
IL	Interleucina
IP-10	Proteína inflamatoria de 10-kDa
ITAC	Quimioattractor α inducible en células T por γ -IFN
JAK	Janus cinasa
LFA-1	Integrina CD11a/CD18 (α L/ β 2)
LPS	Lipopolisacárido
MoAb	Anticuerpo monoclonal
NK	Asesina natural (célula)
PBMC	Células mononucleares de sangre periférica
PI3K	Cinasa de fosfatidilinositol
PRL	Prolactina
PRLR	Receptor de PRL
PT	Toxina pertúsica
PY	Fosfotirosina
RA	Artritis Rheumatoide
SI	Sistema Inmunológico
SLE	Lupus Eritematoso Sistémico
STAT	Transductor de señal y activador de la transcripción
VCAM-1	Molécula de adhesión vascular
VLA-4	Integrina CD49d/CD29 (α 4/ β 7)

ABSTRACT

Attachment of leukocytes to endothelial cells is an essential step for the extravasation and recruitment of cells at sites of inflammation. The pituitary hormone prolactin (PRL) is involved in the inflammatory process. Here, it is shown that treatment with PRL of human peripheral blood mononuclear cells (PBMC) stimulates their adhesion to human umbilical vein endothelial cells (HUVEC) activated by interleukin-1 β . Stimulation of adhesion by PRL is mediated by integrins, leukocyte functional antigen-1 (LFA-1) and very late antigen-4 (VLA-4), because immunoneutralization of both integrins prevents PRL action. Also, PRL promotes the adhesion of PBMC to immobilized intercellular adhesion molecule-1 and fibronectin, ligands for LFA-1 and VLA-4, respectively. Stimulation of integrin-mediated cell adhesion by PRL may involve the activation of chemokine receptors, because PRL upregulates the expression of the G-protein-coupled chemokine receptor CXCR3 in PBMC, and pertussis toxin, a specific G-protein inhibitor, blocks PRL stimulation of PBMC adhesion to HUVEC. In addition, PRL stimulates tyrosine phosphorylation pathways leading to leukocyte adhesion. PRL triggered the tyrosine phosphorylation of Janus kinase-2, of signal transducer and activator of transcription-3 and 5, and of the focal adhesion protein paxillin. Furthermore, genistein, a tyrosine kinase inhibitor, blocked PRL-stimulated adhesion of PBMC and Jurkat T-cells to HUVEC. These results suggest that PRL promotes integrin-mediated leukocyte adhesion to endothelial cells via chemokine receptors and tyrosine phosphorylation signaling pathways.

RESUMEN

La unión de leucocitos a células endoteliales es un paso esencial para la extravasación y reclutamiento de células inmunes hacia los sitios de inflamación. La hormona de la hipófisis anterior, prolactina (PRL) está involucrada en el proceso inflamatorio. En este trabajo, se muestra que el tratamiento de células mononucleares de sangre periférica (PBMC) humanas con PRL estimula su adhesión a células endoteliales de la vena del cordón umbilical (HUVEC) activadas con IL-1 β . La estimulación de la adhesión inducida por PRL es mediada por las integrinas antígeno asociado a la función del linfocito (LFA-1) y antígeno muy tardío (VLA-4), dado que su inmunoneutralización bloquea el efecto de PRL. Asimismo, la PRL promueve la adhesión de las PBMC a molécula de adhesión intercelular-1 (ICAM-1) y fibronectina (FN) inmovilizadas, ligandos de LFA-1 y VLA-4, respectivamente. Este efecto de la PRL sobre la adhesión celular parece involucrar la activación de receptores de quimiocinas, porque la PRL aumenta en las PBMC la expresión del receptor a quimiocinas CXCR3 acoplado a proteínas G, y porque la toxina pertúsica, un inhibidor de proteínas G, bloquea la estimulación por PRL de la adhesión de PBMC a HUVEC. Adicionalmente, la PRL estimula vías de fosforilación de tirosina que llevan a la adhesión de leucocitos. La PRL dispara la fosforilación de tirosinas de cinasa Janus 2 (JAK2), de los transductores de señal y activadores de la transcripción STAT3 y STAT5 y de la proteína de las adhesiones focales paxilina. Más aún, el uso de genisteína, un inhibidor de tirosina cinasas, bloquea la adhesión estimulada por PRL de PBMC y de células Jurkat a HUVEC. Estos resultados muestran que la PRL promueve la adhesión de leucocitos mediada por integrinas a células endoteliales probablemente a través de promover la acción de quimiocinas y la activación de la fosforilación de tirosinas de moléculas mediadoras relevantes.

INTRODUCCIÓN

La hormona prolactina (PRL) se ha asociado a lo largo de la historia principalmente a la función reproductiva. En particular, a los efectos inicialmente descritos sobre el desarrollo de la glándula mamaria y la secreción de leche en mamíferos. Sin embargo, en las últimas décadas se ha descubierto que esta hormona tiene una amplia diversidad de funciones, ya que tiene efectos sobre células de tejidos y órganos más allá del sistema reproductor.

Los efectos de la PRL sobre las células del sistema inmune son sólo un ejemplo que pone de manifiesto la diversidad funcional que esta hormona posee. Hoy en día es ampliamente reconocida la capacidad de PRL para regular la proliferación y función de diferentes poblaciones de leucocitos a través de la activación de su receptor, el cual se encuentra ampliamente distribuido en las células del sistema inmune (SI). Sin embargo, la posibilidad de que la PRL regule la adhesión de leucocitos a endotelio no ha sido explorada. No obstante, esta interacción es muy importante para la función inmune, ya que es un paso fundamental que permite el movimiento de leucocitos del sistema circulatorio a los diversos tejidos y órganos.

La posibilidad de que la PRL regule la adhesión de los leucocitos al endotelio, se deriva de observaciones previas hechas con diferentes citocinas y hormonas, como interleucina-2 (IL-2) y la hormona de crecimiento (GH), con las cuales la PRL comparte diversas características estructurales y funcionales. La IL-2 y la GH promueven la adhesión de leucocitos, mediante la activación de diferentes transductores de señal, algunos de ellos también activados por el receptor de PRL (PRLR). La regulación de la adhesión de leucocitos a endotelio por PRL podría ser relevante en el desarrollo y/o progresión de enfermedades autoinmunes como la artritis reumatoide (RA) y el lupus eritematoso sistémico (SLE), las cuales se han correlacionado con niveles altos de PRL en suero. Así mismo, esta acción de PRL podría ser resultado de una acción hormonal local, debido a que ambos tipos celulares producen PRL. En este contexto, el objetivo del presente trabajo fue explorar la posibilidad de que la PRL regule la adhesión de leucocitos a endotelio vascular, así como analizar algunos de los mecanismos celulares capaces de mediar esta acción.

CAPÍTULO 1

TRÁFICO DE LEUCOCITOS

Las células del sistema inmune se encargan de vigilar constantemente la presencia de antígenos en el organismo, lo que permite iniciar la respuesta inmunológica cuando alguno es encontrado. Esta tarea de vigilancia (“surveillance”) que llevan a cabo los leucocitos se debe en gran medida a su capacidad de extravasación, la cual permite el movimiento continuo de leucocitos entre el torrente sanguíneo y los tejidos. La extravasación leucocitaria puede generar la acumulación de células en los sitios de daño o infección durante procesos inflamatorios, permitiendo así la eliminación de los agentes antigénicos (Springer, 1994; Roitt, et al., 1996). Existen diferentes enfermedades como el cáncer, el síndrome de filtración capilar, el SIDA y otras en las que el proceso de migración de leucocitos se encuentra alterado, lo cual pone en evidencia la importancia que tiene este proceso en la homeostasis del organismo (Mier, et al., 1989; Clauss, et al., 1990; Ohkubo, et al., 1991; Vaillant, et al., 1993; Dhawan, et al., 1995; Jain, et al., 1996). En la siguiente sección se describe el modelo actual que explica la extravasación de leucocitos y que permite entender su especificidad.

MODELO DE CÓDIGO DE ÁREA DE TRÁFICO DE LEUCOCITOS

A mediados de la década pasada se describió el modelo conocido como código de área (“area code”), que explica, con base en las moléculas involucradas en este proceso, los diferentes patrones de migración de leucocitos a los diferentes tejidos (Fig. 1) (Springer, 1994). Este modelo consta de tres etapas: en la primera de ellas, el leucocito circulante reduce su velocidad gracias a la unión de selectinas presentes en su membrana a glicoproteínas de la membrana del endotelio, lo que permite el rodamiento y sujeción (“tethering”) del leucocito en la superficie endotelial (Springer, 1994). La segunda etapa se inicia después de que los leucocitos han reducido su velocidad y se

encuentran en contacto con el endotelio. Esta etapa se caracteriza por la participación de moléculas quimiotácticas

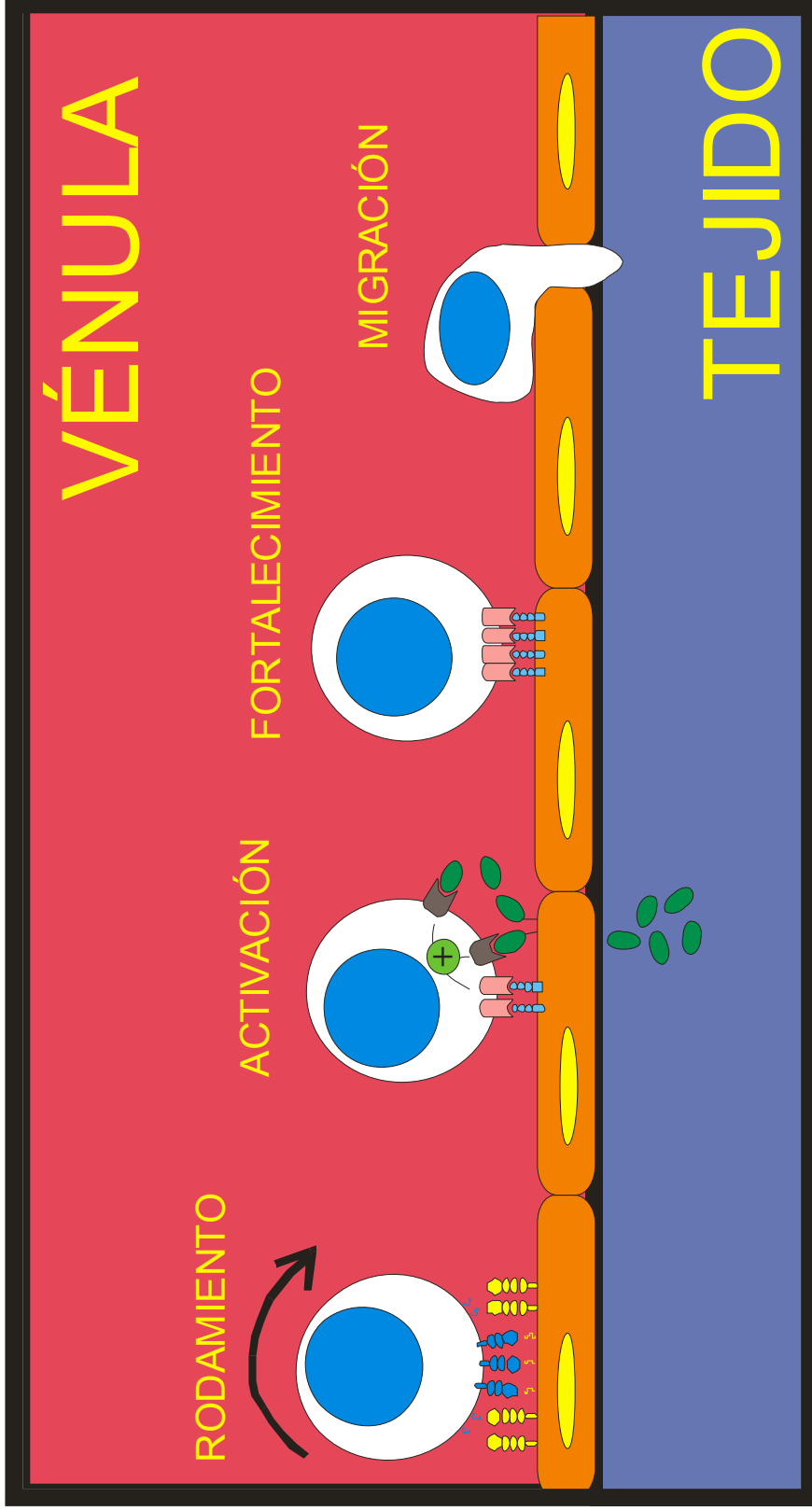


FIGURA 1. Modelo de código de área del tráfico de leucocitos. En el esquema se muestran las etapas que permiten la migración de leucocitos al espacio extravascular, así como las diferentes moléculas involucradas. Durante estos tres pasos las CE y los leucocitos participan activamente en el proceso mediante la expresión, secreción y activación de las diferentes moléculas (Modificada de Roitt, Brostoff et al. 1996).

presentes en la matriz extracelular (ECM), producidas en respuesta a mediadores de inflamación por el endotelio, las células del tejido circulante o las células inmunes. Estos mensajeros moleculares inician cascadas de señales en el leucocito que estimulan la adhesión y regulan la expresión de diferentes genes (Springer, 1994). En la tercera etapa del modelo, la unión entre los dos tipos celulares se fortalece gracias a la redistribución de integrinas en la membrana celular en adhesiones focales (AF) o placas de adhesión (Springer, 1994; Petit and Thiery, 2000). Las AF son estructuras que forman moléculas del citoesqueleto, proteínas adaptadoras e integrinas que median la unión firme entre el leucocito y el endotelio, y cuya regulación intracelular determina la migración de los leucocitos al tejido (Petit and Thiery, 2000). Recientemente se ha propuesto una formalización al modelo del código de área introduciendo la noción de concurrencia (“concurrency”), que implica que las diferentes etapas del proceso se llevan a cabo de manera simultánea y no secuencial. Esta noción permite además explicar cómo la variación cuantitativa de los parámetros pro-adhesivos puede controlar la especificidad y sensibilidad de este proceso (D'Ambrosio, et al., 2004). El patrón de movimiento de granulocitos, linfocitos y monocitos hacia los diferentes tejidos es específico y depende de la subpoblación celular y tejido del que se trate. De acuerdo al modelo de este proceso, la especificidad del patrón de migración de las células se debe al tipo de selectinas, integrinas y receptores a quimioattractores presentes en la membrana de los leucocitos, así como de los quimioattractores y moléculas de adhesión expresadas por el endotelio (Springer, 1994). Para entender por qué las células inmunes presentan diferentes patrones de migración, debe considerarse que las moléculas relacionadas con el tráfico de leucocitos se expresan de manera diferencial dependiendo del tipo de leucocito o endotelio que se trate (Springer, 1994; Ebnet, et al., 1996; D'Ambrosio, et al., 2004). En este sentido, D'Ambrosio y colaboradores proponen la asignación de valores cuantitativos a variables como la redundancia molecular, los niveles de expresión de los diferentes receptores, la velocidad del leucocito, etc. De acuerdo a estos autores, esta asignación numérica permitiría el desarrollo de programas de cómputo que integren dichos valores y que simulen y predigan los patrones de

migración de leucocitos. En las secciones siguientes se describen más ampliamente las características estructurales y funcionales de las moléculas que permiten la salida del leucocito del espacio vascular mediante su interacción con las células endoteliales (CE).

SELECTINAS

Las selectinas son glicoproteínas de membrana que median interacciones célula-célula en procesos como la trombosis, la inflamación crónica, el daño por reperfusión-isquemia y el tráfico de leucocitos. Hasta hoy se han descrito tres diferentes selectinas: la selectina-P presente en plaquetas y en CE, la selectina-E inducible en plaquetas y en CE, y la selectina-L expresada por leucocitos circulantes (Varki, 1997). Las selectinas comparten un patrón estructural que incluye un dominio lectina “tipo C” NH₂-terminal cuya adhesión es dependiente de Ca⁺⁺, un dominio relacionado al factor de crecimiento epidérmico (EGF), un número variable de dominios cortos repetidos conservados, un dominio transmembranal de un solo paso y una cola COOH-terminal citoplásmica (Varki, 1997; Rosen, 1999). La evidencia acumulada señala que las selectinas reconocen carbohidratos de glicoproteínas fucosiladas, sialiladas o sulfatadas. La unión de selectinas a sus sustratos es específica y de baja afinidad (mM), sin embargo un aumento en la afinidad (nM) se logra mediante el reconocimiento de múltiples sitios o por la agregación de selectinas en la membrana (Varki, 1997).

La selectina-L está involucrada principalmente en el proceso de rodamiento y atado de leucocitos a endotelio, aunque se ha encontrado que la selectina-E, inducida en el endotelio por moléculas proinflamatorias, también participa en este proceso (Rosen, 1999). Los ligandos conocidos de la selectina-L son GlyCAM-1, CD34, PSGL-1, Sgp200, y podocalixina, presentes en la membrana de las CE. Estos ligandos requieren ser sialilados, fucosilados o sulfatados de manera específica para ser reconocidos por esta selectina (Rosen, 1999). La unión de la selectina-L a sus ligandos no sólo permite el rodamiento y atado de leucocitos, sino además inicia cascadas intracelulares que activan integrinas $\beta 1$ y $\beta 2$, lo que permite la unión firme de leucocitos a endotelio o a moléculas de la ECM (Hwang, et al., 1996; Giblin, et al., 1997).

MOLÉCULAS QUIMIOTÁCTICAS

Las moléculas quimiotácticas poseen la característica de atraer leucocitos mediante la inducción de movimiento celular en función de su gradiente de concentración, proceso conocido como quimiotaxis. Estas moléculas se agrupan en dos diferentes conjuntos: los quimioattractores clásicos (p. ej. C5a, leucotrieno B4, etc.) y las quimiocinas (IL-8, IP-10, MIP-1, etc) (Roitt, et al., 1996). Los quimioattractores clásicos son moléculas heterogéneas que bien pueden ser secretadas por células o generadas por procesamiento enzimático. El leucotrieno B4 es producido por macrófagos activados, mientras que C5a o los fibrinopéptidos se generan por la activación de la cascada del complemento o del sistema de coagulación (Roitt, et al., 1996).

QUIMIOCINAS

El grupo de quimiocinas humanas comprende más de 40 moléculas que comparten características estructurales y funcionales definidas inicialmente para la IL-8. Éstas son proteínas quimiotácticas pequeñas (de 8-14 kDa) con 4 cisteínas conservadas que forman dos puentes disulfuro (Baggiolini, 1998; Rot and von Andrian, 2004). Las quimiocinas se agrupan en quimiocinas CC, CXC, de acuerdo a la posición de las dos primeras cisteínas que pueden ser adyacentes o estar separadas por un aminoácido. Varias quimiocinas se producen en condiciones patológicas por las células de los tejidos y los leucocitos infiltrados, lo que promueve el tráfico de células inmunes a los sitios de inflamación. Además, estas moléculas regulan procesos celulares como la proliferación, la maduración, la apoptosis, la transformación maligna y la diseminación (Rot and von Andrian, 2004). Estas moléculas juegan un papel en el proceso de maduración de leucocitos en la médula ósea, la renovación de leucocitos circulantes y algunas quimiocinas tienen efectos sobre células del sistema nervioso central y las CE (Baggiolini, 1998; Murdoch and Finn, 2000; Rossi and Zlotnik, 2000).

Las quimiocinas son producidas por una gran diversidad de células, y su efecto es mediado por la unión a sus receptores. De éstos se han encontrado 6 para quimiocinas CXC y 8 para quimiocinas CC, todos poseen siete dominios transmembranales y están acoplados a proteínas G. Estos receptores se expresan de distinta manera en las diferentes poblaciones de leucocitos, lo cual permite que su migración sea tejido-específica, debido a las diferencias de expresión de sus ligandos en los tejidos (Murdoch and Finn, 2000; Rot and von Andrian, 2004). Las quimiocinas y sus receptores tienen alta redundancia, esto se debe a que la mayoría de los receptores pueden reconocer a más de una quimiocina, o bien que diversas quimiocinas pueden activar a más de un receptor (Baggiolini, 1998; Rossi and Zlotnik, 2000; Rot and von Andrian, 2004).

Las quimiocinas son moléculas solubles que pueden ser retenidas en la ECM mediante su unión a glicosaminoglicanos, lo que puede favorecer la activación de su receptor cuando el leucocito rueda sobre el endotelio (Ebnet, et al., 1996; Rot and von Andrian, 2004). Esta característica de unión a glicosaminoglicanos de las quimiocinas

evita su difusión y permite además la orientación del leucocito en la ECM (Rot and von Andrian, 2004). La unión de las quimiocinas a sus receptores en leucocitos activa la cascada de señales de proteínas G, lo que entre otras cosas permite el aumento de afinidad de las integrinas así como de su avidéz, por el rearrreglo de la integrinas en la membrana. Estos cambios de afinidad y avidéz permiten la adhesión firme de leucocitos a endotelio, su desplazamiento y extravasación (Ebnet, et al., 1996; Murdoch and Finn, 2000; Rot and von Andrian, 2004). Adicionalmente, se ha encontrado que las quimiocinas también generan la redistribución de las moléculas de adhesión celular de las CE (explicada más adelante), favoreciendo así la extravasación de leucocitos (del Pozo, et al., 1997). La señalización de los receptores a quimiocinas ocurre a través de GTPasas de la familia RAS (RAP1, RAPL, RHOA), proteínas del citoesqueleto (talina y paxilina), cinasas de serina (proteína cinasa atípica- ζ , fosfoinositido-3 cinasa [PI3K]) y tirosina cinasas (pyk2, FAK) (Imhof and Aurrand-Lions, 2004; Rot and von Andrian, 2004).

INTEGRINAS

Las integrinas son moléculas de adhesión formadas por dos subunidades asociadas no covalentemente (α y β) que atraviesan la membrana celular. Estas moléculas de adhesión son probablemente las más versátiles, pues existen 16 subunidades α y 8 β que se combinan en 22 dímeros diferentes que permiten la adhesión celular a diferentes sustratos. Estas moléculas se agrupan en tres categorías principales dependiendo de la subunidad β que posean y de su función, aunque existen algunas excepciones en estas categorías. Las integrinas β_1 suelen estar relacionadas con la unión de las células a la ECM; las integrinas β_2 y β_3 con la adhesión célula a célula, en particular las β_2 median la adhesión de leucocitos a endotelio y otras células inmunes, mientras que las β_3 participan en las interacciones de plaquetas y neutrófilos en los sitios de inflamación o daño vascular. (Krissansen, 2001).

La unión de las integrinas a sus ligandos se modula de dos formas principalmente: regulando su afinidad y su avidéz. La afinidad se refiere a la fuerza de unión entre la integrina y su ligando, y es regulada mediante cambios conformacionales propagados desde el dominio intracitoplásmico de la integrina hasta los dominios

extracitoplásmicos de unión. Por su parte, la avidéz se refiere a la suma de las afinidades de las integrinas, y es regulada por señales intracelulares que remodelan la estructura del citoesqueleto y sus interacciones, así como las tasas de difusión de las integrinas en la membrana (Hughes and Pfaff, 1998; Carman and Springer, 2003; Imhof and Aurrand-Lions, 2004). Los cambios conformacionales de las integrinas se deben a cascadas intracelulares en las que participan fosfolipasa C (PLC), proteína cinasa C (PKC), R-Ras, Raf, Rho, la proteína cinasa activada por mitógenos (MAPK) y la cinasa de fosfoinosítido-3 (PI3K) entre otras (Hughes and Pfaff, 1998; Epler, et al., 2000). Los eventos que activan estas cascadas de señales son diversos e incluyen la activación del receptor de célula T (TCR) así como la activación de receptores a quimiocinas u otras citocinas (Hughes and Pfaff, 1998; Imhof and Aurrand-Lions, 2004). La unión de las integrinas a su ligando inicia además cascadas de señales intracelulares que involucran moléculas como FAK, Abl y Syk entre otras y que regulan la función celular (Schlaepfer and Hunter, 1998).

Las integrinas que principalmente participan en el tráfico de leucocitos son LFA-1 (CD11/CD18 o α L/ β 2) y VLA-4 (CD23/CD49 o α 4/ β 1), las cuales se encuentran ampliamente distribuidas en diferentes poblaciones de leucocitos. Existen algunas otras integrinas que median la adhesión a endotelio como LPAM-1 (α 4/ β 7) y Mac-1 (α M/ β 2). Sin embargo, éstas se expresan sólo en algunas subpoblaciones de leucocitos (Springer, 1994; Ebnet, et al., 1996). La regulación secuencial, coordinada de la unión de las integrinas a sus ligandos así como la construcción y deconstrucción de las adhesiones focales (AF), generan sitios de mayor adhesividad en la membrana de la célula, lo que permite el desplazamiento orientado de los leucocitos al espacio extravascular (Dustin and Springer, 1991; Springer, 1994; Bianchi, et al., 1997; Imhof and Aurrand-Lions, 2004). Las integrinas LFA-1 y VLA-4 se unen a moléculas de adhesión celular que se inducen en el endotelio por mediadores de inflamación; LFA-1 reconoce ICAM-1 y VLA-4 VCAM-1. Adicionalmente, VLA-4 interacciona con fibronectina (FN) (Dustin and Springer, 1991; Springer, 1994; Ebnet, et al., 1996). En la siguiente sección se explican las características estructurales y funcionales de las moléculas de adhesión celular a las que se unen las integrinas.

MOLÉCULAS DE ADHESIÓN CELULAR

Las moléculas de adhesión celular (CAMs) pertenecen a la superfamilia de las inmunoglobulinas. La mayoría de estas moléculas son expresadas o inducibles en el endotelio y otros tipos celulares por los diferentes mediadores de inflamación como el factor de necrosis tumoral- α (TNF- α) o la interleucina-1 β (IL-1 β) (Springer, 1994; Ebnet, et al., 1996; Roitt, et al., 1996). Las moléculas de adhesión celular involucradas directamente en el tráfico de leucocitos son ICAM-1, ICAM -2 e ICAM-3 a las que se unen las integrinas β 2 LFA-1 y Mac-1; VCAM-1 a la que se une la integrina β 4 VLA-1; y MadCAM-1 a la que se une la integrina LPAM-1. MadCAM-1 también actúa como ligando-mucina para selectina-L (Springer, 1994; Ebnet, et al., 1996; Varki, 1997). ICAM-2 e ICAM-3 son expresadas de manera constitutiva en el endotelio, mientras que ICAM-1 y VCAM-1 son inducibles por citocinas proinflamatorias y lipopolisacáridos de origen bacteriano en el endotelio (Springer, 1994; Ebnet, et al., 1996). MadCAM-1 por su parte es inducible de manera específica en el endotelio de los parches de Peyer (Peyer's patches), lo que explica la migración preferencial de ciertas poblaciones de linfocitos a estos tejidos linfáticos secundarios (Rosen, 1999).

ADHESIONES FOCALES

La adhesión de células a la ECM es crucial, no sólo en el tráfico de leucocitos, sino además en otros procesos biológicos como la diferenciación y la proliferación celular. Cuando las células se unen por medio de integrinas a la ECM se forman estructuras en regiones especializadas de la membrana plasmática llamadas AF. En estos sitios, los haces ("bundles") de filamentos de actina se anclan a integrinas membranales a través de complejos multi-moleculares o proteínas de la placa de unión. Algunas de las moléculas de las AF participan en la unión estructural entre las integrinas y el citoesqueleto de actina, mientras que otras son moléculas de señalización, entre ellas cinasas y fosfatasas, o bien sustratos de las cinasas respectivas o proteínas adaptadoras (Fig. 3). Se sabe que la unión de las integrinas dispara eventos de transducción de señales como la fosforilación de tirosinas, la elevación del pH celular, o la activación de la cascada de MAPK. Estas cascadas de señales regulan la formación de las AF, permitiendo la migración del leucocito gracias a la activación secuencial de eventos funcionales dependientes de la adhesión celular (Burrige and Chrzanowska-Wodnicka, 1996; Bianchi, et al., 1997; Petit and Thiery, 2000).

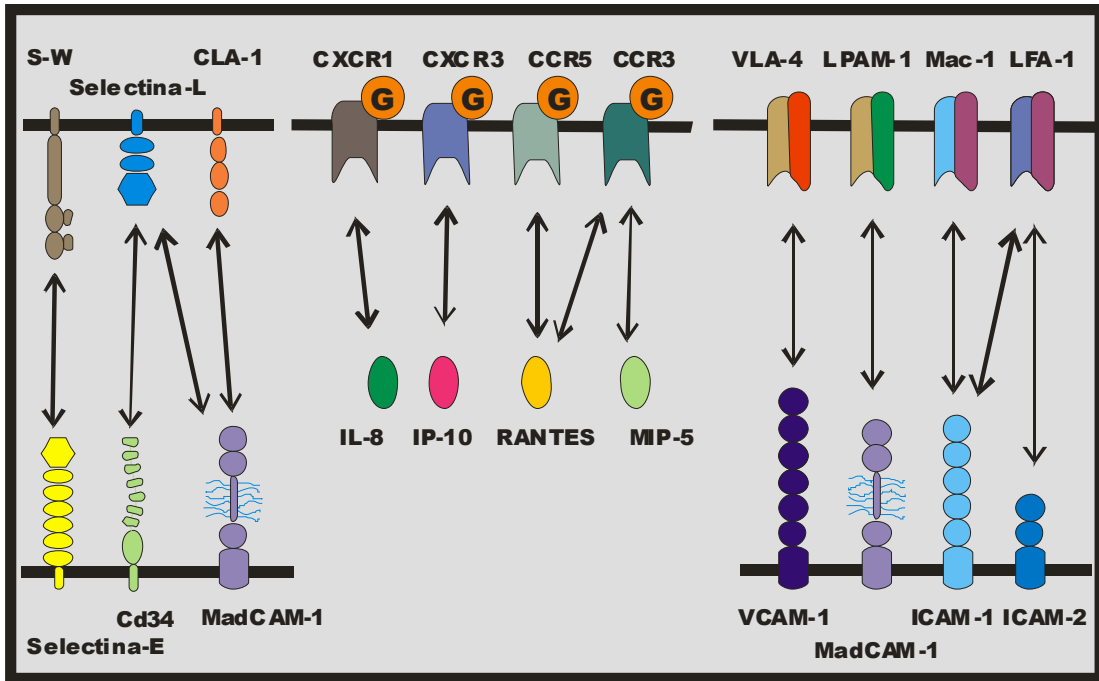


Figura 2. Algunas de las moléculas que intervienen en el tráfico de leucocitos. Según el modelo de código de área un leucocito rodará sobre el endotelio que exprese CD34 u otro ligando que reconozca selectina-L. Si el endotelio produce IP-10, sólo los leucocitos con receptores CXCR3 favorecerán su interacción con el endotelio. Finalmente, si el endotelio expresa MadCAM-1 sólo los leucocitos que expresen LPAM-1 migrarán hacia el tejido. De esta manera las subpoblaciones de leucocitos migran diferencialmente dependiendo del tipo de moléculas que expresan ambos tipos celulares (Figura tomada de Springer, 1994).

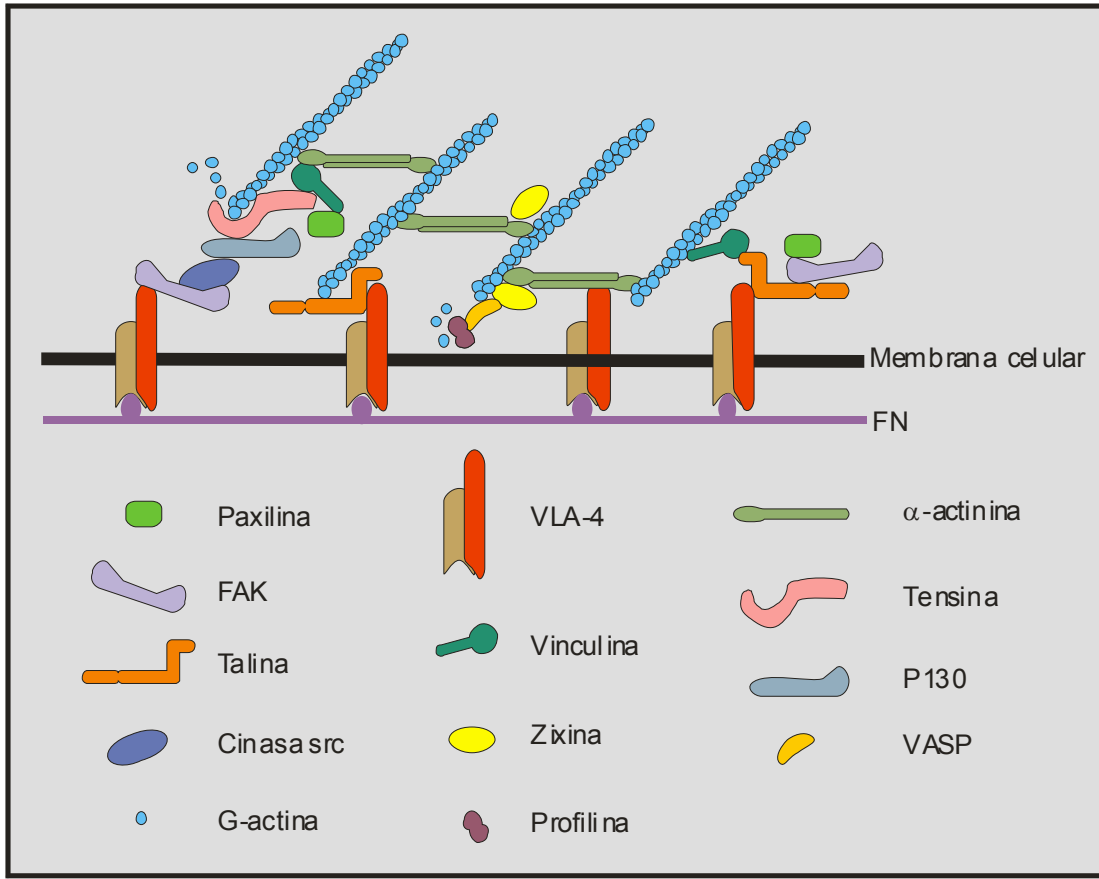


Figura 3. Esquema de las adhesiones focales. En el esquema se muestran algunas de las interacciones entre moléculas de las adhesiones focales formadas en la membrana de una célula adherida a una superficie cubierta con FN (Tomada de Burridge y Chrzanowska-Wodnicka, 1996).

CAPÍTULO 2

PROLACTINA

Hace ya varios años se reconoció la interacción existente entre las células de los sistemas inmune, nervioso y endócrino, de tal forma que hoy se habla de un sólo sistema integral llamado neuroinmunoendócrino. Esta integración hace referencia a la capacidad que tienen las células de cada uno de estos sistemas para regular mutuamente su actividad y función a través de diferentes mensajeros. Por ejemplo, está bien establecido que el estrés puede modificar la inmunocompetencia de los organismos; que péptidos secretados por los leucocitos pueden regular la secreción de hormonas por parte de las glándulas endócrinas, o bien que las hormonas pueden funcionar como reguladoras de los leucocitos, de la actividad neuronal y de la conducta (Spangelo, et al., 1989; Glaser, et al., 1990; Murphy, et al., 1995; Berczi, 1997).

Hace ya varias décadas se encontraron las primeras evidencias de que las hormonas hipofisarias tienen un papel en la regulación de la respuesta inmune. Smith en 1930 demostró que la pituitaria anterior es importante para mantener la integridad del timo (Smith, 1930), y más tarde se obtuvieron evidencias directas de la relación entre la pituitaria anterior y el sistema linfóide (Pierpaoli, 1967; Pierpaoli and Sorkin, 1968). En 1988, Diane Russel reunió diversos reportes sobre el papel de la PRL en la inmunomodulación (Russell, 1988). En las siguientes secciones se describen aspectos generales de la PRL y su receptor (PRLR), y se revisa el papel de esta hormona en la regulación del sistema inmune.

La PRL es una hormona proteica descubierta hace más de 60 años (Riddle, et al., 1932). Esta hormona es codificada por un solo gen, que en el humano se localiza en el cromosoma 6 y cuenta con 5 exones y 4 intrones. En su forma nativa la PRL tiene una masa molecular de 23 kDa y consta, de acuerdo a la especie, de alrededor de 200 residuos de aminoácidos dispuestos en cuatro α -hélices antiparalelas (Fig. 4). Se han descrito diferentes variantes moleculares de PRL que son producto de modificaciones postranscripcionales (procesamiento alternativo del mRNA) o postraduccionales (proteólisis, desaminación, glicosilación, fosforilación, sulfatación, agregación, etc.) (Sinha, 1995; Bole-Feysot, et al., 1998; Freeman, et al., 2000). Una de las variantes

mejor conocidas es la PRL cortada que fue descrita inicialmente por Mitra (Mitra, 1980). Esta variante presente en la rata y el humano puede generarse mediante la proteólisis enzimática entre la tirosina 145 y la leucina 146. La reducción del puente disulfuro intermedio de la PRL cortada la rompe en dos fragmentos: uno de 16 kDa correspondiente al extremo N-terminal y otro de 7 kDa correspondiente al extremo C-terminal (Mitra, 1980; Clapp, 1987; Sinha, 1995).

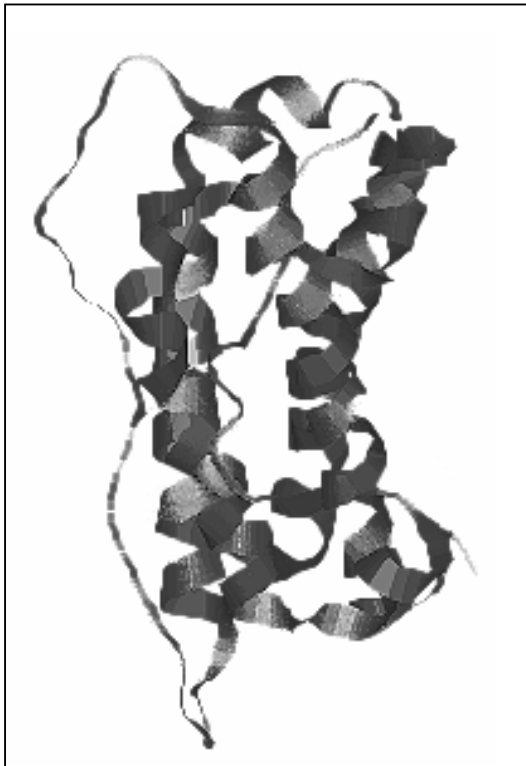


Figura 4. Representación de listón de la estructura tridimensional de PRL, modelada con base en estudios cristalográficos de la hormona de crecimiento

La PRL es producida y secretada principalmente por la adenohipófisis, aunque también se han reportado diversos sitios extrahipofisarios en los que esta hormona se produce, que incluyen la placenta, el útero, el cerebro, el sistema inmune, la próstata, la glándula lacrimal y el endotelio vascular, entre otros (Sinha, 1995; Ben-Jonathan, et al., 1996; Wu, et al., 1996; Clapp, et al., 1998; Freeman, et al., 2000). Los efectos más conocidos de esta hormona son los que ejerce sobre la glándula mamaria y el sistema reproductor, sin embargo también ejerce efectos sobre la osmorregulación, el crecimiento, la inmunomodulación y la angiogénesis entre otros (Bole-Feysot, et al., 1998; Clapp, et al., 1998; Corbacho, et al., 2002).

EL RECEPTOR DE PROLACTINA

La amplia gama de efectos que lleva a cabo la PRL es mediada por receptores transmembranales de los que existen diferentes isoformas moleculares. Los receptores a PRL (PRLR) pertenecen a la superfamilia de receptores para citocinas/hematopoyetina que incluyen los receptores para GH, IL-2 e IL-7 (IL-2 β y γ), y el factor estimulante de colonias de granulocitos-macrófagos (GM-CSF) entre otros. Esta superfamilia de receptores se caracteriza por tener dos dominios extracelulares (en los que se encuentran 4 cisteínas conservadas y el motivo triptófano-serina o WS) y dos regiones intracelulares conservadas llamadas caja 1 y caja 2. Hasta hoy se han descrito tres isoformas del receptor de PRL, que resultan del procesamiento alternativo del mRNA: el largo, el intermedio y el corto, los cuales varían en el tamaño de su región intracelular. Además, en algunos fluidos orgánicos se ha detectado una forma soluble del receptor producto de un procesamiento enzimático del receptor membranal (Postel-Vinay, 1996; Wells and de Vos, 1996; Bole-Feysot, et al., 1998; Clevenger, et al., 1998).

Para que el PRLR sea activado es necesario que la PRL interactúe con dos receptores a la vez, formando un dímero de dos receptores unidos a una molécula de PRL. Esto implica que la PRL posee dos sitios de unión al receptor. La unión entre el receptor y la hormona se establece cuando la PRL es reconocida en su sitio de unión 1 por un primer receptor y, posteriormente la misma molécula es reconocida en su sitio de unión 2 por un segundo receptor. A este mecanismo de activación del PRLR se le conoce como el modelo de activación por dimerización (Fig. 5) (Fuh, et al., 1993; Bole-Feysot, et al., 1998; Clevenger, et al., 1998).

La señalización del PRLR incluye la activación de diferentes cinasas capaces de fosforilar diferentes sustratos intracelulares, entre las que sobresalen las cinasas de la familia Janus (JAKs), en particular JAK2 que es activada por las tres isoformas del receptor clonado (Clevenger and Kline, 2001); los transductores de señal y activadores de transcripción 1, 3 y 5 (STATs), que pueden ser activados por JAK2, y las cinasas de proteínas activadas por mitógenos (MAPK) entre otras (Bole-Feysot, et al., 1998; Wells y de Vos, 1996). La activación de estos transductores tiene como resultado a su vez la activación de distintos sustratos intracelulares que son capaces de regular la expresión de diferentes genes (Yu-Lee, et al., 1998). También se ha encontrado que la PRL activa

la vía de la cinasa de las adhesiones focales (FAK), la cual se localiza en las AF y ha sido implicada en la migración y adhesión celular (Canbay, et al., 1997). De hecho, la vía de JAK y STATs ha sido propuesta como activadora de FAK en respuesta a hormona de crecimiento (Ryu, et al., 2000). La activación de FAK resulta en la fosforilación de diversos componentes de las AF, uno de ellos es paxilina, molécula adaptadora que provee una plataforma para la integración y procesamiento de señales relacionadas a la adhesión y a factores de crecimiento (Turner, 2000). Se ha encontrado que la PRL estimula la fosforilación/activación de paxilina en células de cáncer mamario (Canbay, et al., 1997). En la Fig. 6 se presenta un esquema de las vías de señalización del PRLR y algunos de los genes que regula de manera directa e indirecta.

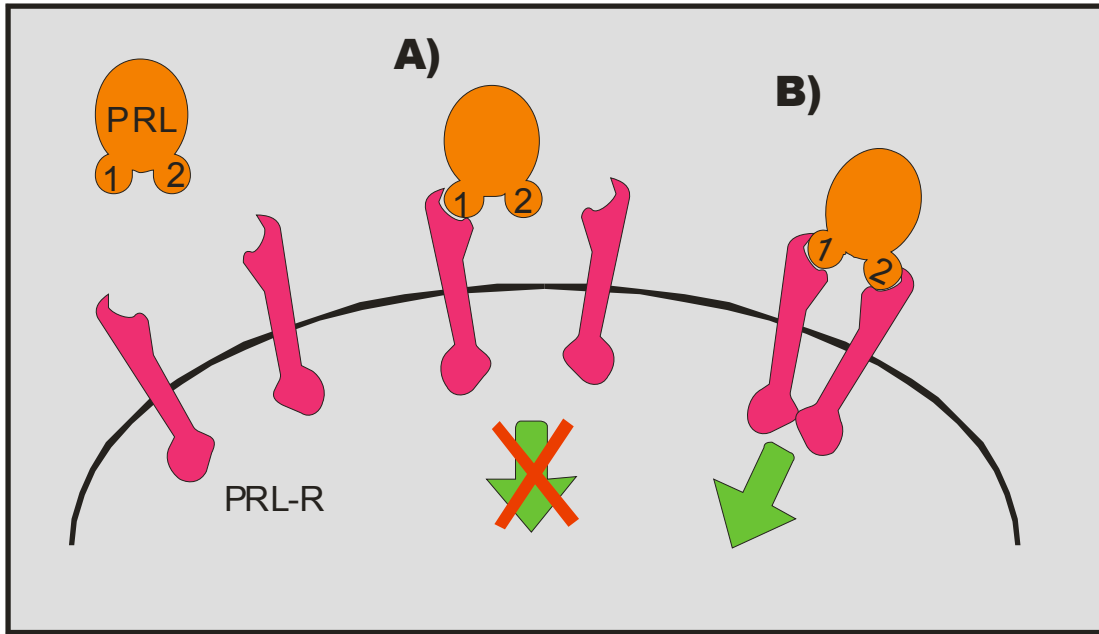


Figura 5. Modelo de activación del PRLR. La activación del PRLR se basa en la dimerización inducida por PRL. En un paso inicial A) la PRL se une a un PRLR mediante su sitio de unión 1, formándose así un complejo hormona-receptor inactivo. Enseguida B) la PRL se asocia mediante su sitio de unión 2 a otro PRLR lo que lleva a la homodimerización y activación de las cascadas intracelulares (Tomado de Bole-Feysot et al. 1998).

Además de los receptores de PRL ya identificados, existen evidencias experimentales que indican la existencia de receptores específicos para el fragmento N-terminal de 16 kDa de la PRL (PRL-16K), que no son activados por otras prolactinas incluyendo la PRL de 23 kDa (Clapp and Weiner, 1992). Aunque su secuencia y mecanismos de señalización son aún desconocidas, las evidencias indican que estos receptores para PRL 16 kDa parecen ser funcional y estructuralmente diferentes a los receptores ya clonados (Clapp and Weiner, 1992). La importancia funcional de estos receptores se basa la observación de que la PRL-16K tiene efectos diferentes a la PRL. Esta variante de PRL inhibe la formación de nuevos vasos sanguíneos (angiogénesis) a través de acciones directas sobre las CE, que incluyen la inhibición de su proliferación y la inducción de su apoptosis (Clapp, et al., 1993; Buckley and Buckley, 2000).

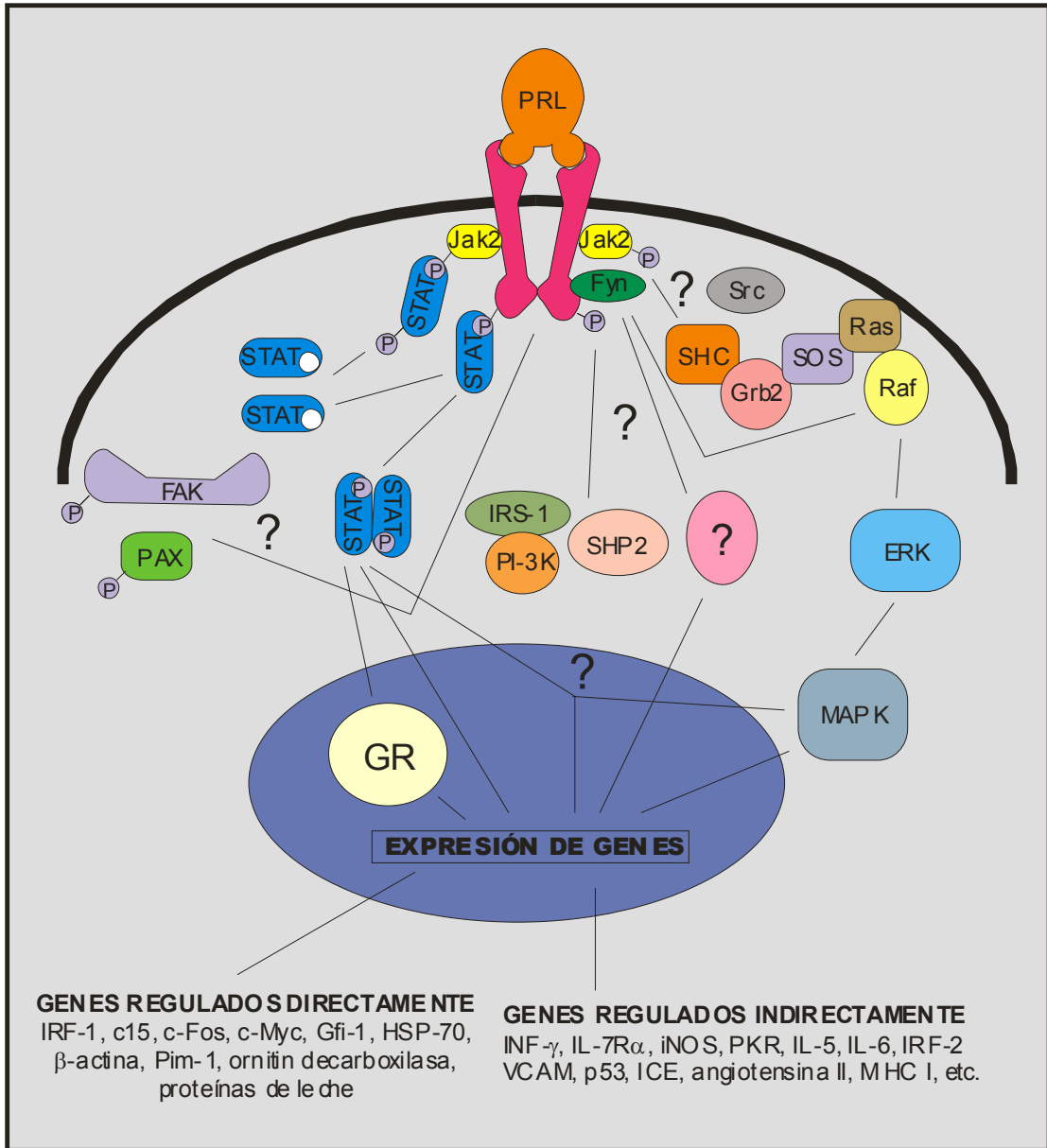


Figura 6. Representación esquemática de las vías de señalización del PRLR. El PRLR activa a Stat 1, Stat 3 y principalmente a Stat 5. La interacción de Stat 5 con el Receptor a Glucocorticoides (GR) ya se ha reportado. La vía de la cinasa MAP involucra la cascada de Shc, Grb2, Sos, Ras y Raf y al parecer también es activada por la isoforma corta del receptor, el cual también activa la vía de Jak 2 y de Fyn. Se han propuesto conexiones entre las vías de Jak, Stat y MAPK. Las interacciones entre el PRLR y cinasas de la familia Src, SHP2, IRS-1, IP-3K y otras moléculas de transducción aún no están claras (Modificado de Bole-Feysot et al., 1998).

LA PROLACTINA Y EL SISTEMA INMUNE

Desde principios de la década de los 80 se han hecho trabajos con diversas especies que han arrojado múltiples evidencias sobre los efectos que tiene la PRL en la regulación de la función inmune. Las primeras evidencias fueron obtenidas en ratas donde la hipofisectomía se asoció con el desarrollo de enfermedades como anemia, leucopenia y trombocitopenia, una respuesta humoral suprimida y ausencia de sensibilidad por contacto a nitroclorobenceno (Berczi, et al., 1983; Nagy, et al., 1983a; Nagy, et al., 1983b; Nagy and Berczi, 1989). De manera similar, el tratamiento de ratas con bromocriptina (BCR) (agonista dopaminérgico que inhibe la secreción hipofisiaria de PRL) reduce la producción de anticuerpos, altera la proporción de marcadores de superficie de linfocitos en ratones neonatos, reduce la activación de macrófagos dependiente de linfocinas y la producción de interferón gamma (INF- γ) por parte del bazo. En estos experimentos la función linfohematopoiética se restablece administrando PRL exógena o injertando hipófisis bajo la cápsula del riñón para generar hiperprolactinemia (Nagy, et al., 1983a; Nagy, et al., 1983b; Hiestand, et al., 1986; Bernton, et al., 1988; Russell, 1988).

En otros experimentos, los investigadores han relacionado el aumento en la PRL circulante con una disminución en los niveles circulantes de IL-2 (Vidaller, et al., 1986) y la disminución de actividad de células naturales asesinas (NK) (Gerli, et al., 1986). Además, todos estos efectos se revierten mediante la administración de BCR, lo que sugiere la participación de la PRL sistémica en todas estas acciones (Gerli, et al., 1986; Vidaller, et al., 1986; Lavalle, et al., 1987). Estos resultados apoyan el papel de PRL como reguladora de la función inmune.

También existen evidencias que involucran a la PRL con la activación de linfocitos T y otras células inmunocompetentes. Se ha observado que sueros anti-PRL bloquean la proliferación inducida por fitohemaglutinina o concanavalina A de linfocitos T humanos y de la línea celular CTLL-2 dependiente de IL-2 (Hartmann, et al., 1989; Sabharwal, et al., 1992). Además, se ha encontrado que la proliferación de linfocitos T, B y células NK en respuesta a diferentes mitógenos puede ser estimulada o inhibida por PRL de manera dosis-dependiente (Matera, et al., 1992). De hecho, se ha propuesto una acción coordinada entre PRL e IL-2 en la respuesta proliferativa de

linfocitos T, en la maduración y actividad citotóxica de células NK y en la generación de células asesinas activadas por linfocinas (LAK cells) (Clevenger, et al., 1990; Matera, et al., 1990; Cesano, et al., 1994). Estas observaciones han originado un modelo de activación en dos pasos para los linfocitos T, en el cual se propone que la PRL y la IL-2 son factores autócrinos necesarios para la progresión de ciclo celular (Fig. 7) (Clevenger, et al., 1992; Cesano, et al., 1994). De hecho, en clara asociación con los efectos de PRL sobre la activación de los linfocitos T, se ha encontrado que el rechazo a trasplantes de corazón (efecto predominantemente debido a linfocitos T) se asocia con un aumento de los niveles circulantes de PRL (Larson, et al., 1985; Carrier, et al., 1987).

En apoyo a los efectos autócrinos de PRL sobre la proliferación de células inmunes se ha reportado que esta hormona es producida por las células Jurkat (línea celular derivada de un linfoma de células T humanas) y que anticuerpos contra PRL inhiben la proliferación de estas células en cultivo (Matera, et al., 1997). Más aún, mediante diversas aproximaciones experimentales, se ha encontrado la expresión de PRL y su receptor en diversas poblaciones de leucocitos, entre las que se cuentan las células mononucleares, los linfocitos, las células NK, los esplenocitos, los timocitos, algunas células de tumores leucémicos y diferentes líneas celulares de origen hematopoiético (U-937, YT, Hut 78, Jurkat, etc.) (Ben-Jonathan, et al., 1996; Wu, et al., 1996; Bole-Feysot, et al., 1998). Asimismo, la expresión de PRL y su receptor se ha detectado en diferentes tejidos linfoides como el bazo, el timo y los nódulos linfáticos, tejidos en los cuales la comunicación intercelular mediada por citocinas es fundamental (Ben-Jonathan, et al., 1996; Wu, et al., 1996; Bole-Feysot, et al., 1998).

Existen también trabajos que sugieren la participación de PRL en la homeostasis del sistema inmune bajo condiciones de estrés, incluyendo procesos de trauma, infección e inflamación. Se ha encontrado que la administración de PRL exógena reduce la mortalidad de animales expuestos a un choque hemorrágico o infectados con *Salmonella*, efecto que al parecer está asociado a la producción de óxido nítrico (NO) (Meli, et al., 1996; Zellweger, et al., 1996a). También se ha reportado que la PRL estimula diferentes parámetros inflamatorios en el modelo experimental de carragenina (Meli, et al., 1993). Así mismo, se ha encontrado que la PRL aumenta la producción del Factor XII (o factor de Haageman) en el hígado, la producción de NO en endotelio, de

NO e IL-1 en macrófagos y de NO y TNF- α en neutrófilos (Gordon, et al., 1991; Kumar, et al., 1997; Meli, et al., 1997). La PRL también restaura la producción de IL-2, IL-3 y la proliferación de esplenocitos después de un shock hemorrágico, regula la expresión de ICAM-1, la acumulación de macrófagos en el cuerpo lúteo, media procesos inflamatorios en la próstata de ratas castradas y aumenta la quimiotaxis y la fagocitosis de macrófagos (Tangbanluekal and Robinette, 1993; Ortega, et al., 1997; Narnaware, et al., 1998; Knoferl, et al., 2000; Olson and Townson, 2000). Recientemente también se reportó que la respuesta de fase aguda inducida por LPS aumenta la expresión del PRLR en el timo (Corbacho, et al., 2004). Finalmente, se ha encontrado que la PRL modula la interacción entre timocitos y células epiteliales del timo estimulando la adhesión entre ambos tipos celulares (de Mello-Coelho, et al., 1997). Todos estos hallazgos han reforzado la propuesta de que la PRL sea considerada una citocina, es decir, un mensajero químico del SI.

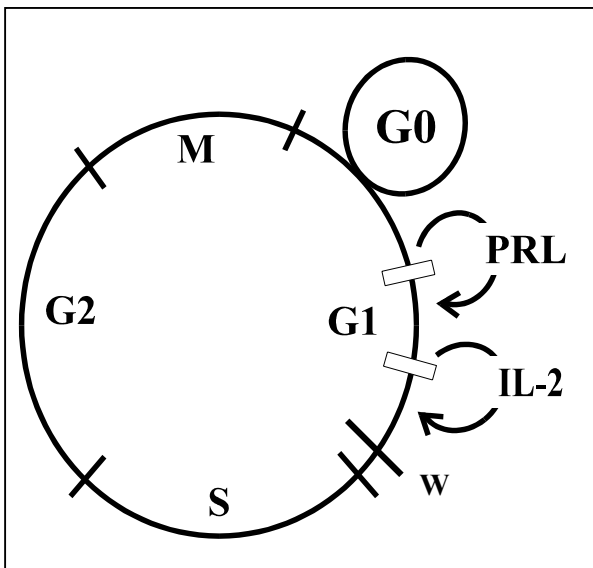


Figura 7. En el ciclo celular de los linfocitos T se propone que PRL e IL-2 son factores autócrinos necesarios para su progresión.

La PRL además se ha relacionado con el desarrollo de enfermedades autoinmunes. Desde hace más de 100 años se conoce la mayor incidencia de lupus eritematoso sistémico (SLE) en mujeres que en hombres (Whitacre, 2001). Hoy en día sabemos que esta relación con el género es común a varias enfermedades autoinmunes como SLE, artritis reumatoide (AR) y esclerosis múltiple (Whitacre, 2001). Por esta razón las hormonas sexuales y algunas hormonas con características dimórficas sexuales como la PRL están siendo investigadas activamente como factores involucrados en el

desarrollo de enfermedades autoinmunes (McMurray, 2001a; Whitacre, 2001). En apoyo a esta consideración, en pacientes con RA, SLE y esclerosis múltiple se han observado niveles altos de PRL en la circulación. Más aún, actualmente se ha propuesto que la PRL podría ser uno de los factores que favorecen la mayor incidencia de SLE y RA en mujeres durante el embarazo (Sercarz and Datta, 1994; Velkeniers, et al., 1998; McMurray, 2001b). Finalmente, la PRL, se ha relacionado con el desarrollo de otras enfermedades como el SIDA y algunas neoplasias como las de mama, colon y próstata (Ginsburg and Vonderhaar, 1995; Velkeniers, et al., 1998).

No obstante la gran cantidad de evidencias que señalan que la PRL juega un papel en la inmunomodulación, esta idea se ha puesto en duda por experimentos realizados en modelos “knock-out” (KO) para la PRL y su receptor. En estos animales genéticamente modificados se ha encontrado que la hematopoyesis, la sobrevivencia a infecciones y otros parámetros de la respuesta inmune no se alteran por la ausencia de esta hormona o su receptor, a diferencia de la función reproductiva que se interrumpe notablemente (Horseman, et al., 1997; Bouchard, et al., 1999; Foster, et al., 2000). Se han propuesto diversas posibilidades que intentan explicar la aparente discrepancia entre los resultados obtenidos en los animales KO para PRL y el RPRL y los hallazgos previamente mencionados en otros modelos experimentales. Una de estas explicaciones se basa en la existencia de mecanismos de compensación debido a la redundancia funcional que existe entre los miembros de la superfamilia de receptores a citocinas (Bouchard, et al., 1999). Otra explicación propone que la PRL no es indispensable para una respuesta inmune funcional, pero que desempeña un papel potenciador de la acción de otras moléculas involucradas en dicha acción. Por lo tanto, la contribución de sus acciones depende de la concertación de otros mediadores del sistema inmune. Recientemente, se ha propuesto que en este sentido las acciones potenciadoras de la PRL son particularmente relevantes bajo condiciones de estrés (Foster, et al., 2000; Dorshkind and Horseman, 2001).

Si bien es claro que esta hormona puede participar a diferentes niveles en la regulación de la respuesta inmune, el papel de PRL en la adhesión de leucocitos a endotelio no se ha explorado, a pesar de que este proceso es fundamental para el establecimiento y la consolidación de la función inmune. Así pues, considerando la importancia que tiene la interacción entre el endotelio y los leucocitos y los reportes que

demuestran la participación de PRL en el proceso inflamatorio, se propone investigar el papel de PRL en la interacción entre leucocitos y endotelio.

HIPÓTESIS

“La PRL regula la adhesión de leucocitos al endotelio vascular.”

OBJETIVOS

OBJETIVO GENERAL

Analizar si la prolactina modifica la adhesión de células mononucleares de sangre periférica (PBMC) al endotelio de la vena umbilical humana (HUVEC).

OBJETIVOS PARTICULARES

1.- Determinar si la PRL recombinante humana regula la adhesión de PBMC a HUVEC.

2.- Investigar algunos de los mecanismos celulares involucrados en la regulación de la adhesión mediada por PRL.

MATERIALES Y MÉTODOS

REACTIVOS Y ANTICUERPOS

Los reactivos empleados se obtuvieron de los siguientes proveedores: la fibronectina (FN) de plasma humano, el suero fetal bovino (FBS), la heparina porcina, la solución de Hank (HBSS), los medios de cultivo M199 y RPMI-1640, la penicilina-estreptomocina, la tripsina-EDTA, la L-glutamina, la fungizona y la Taq polimerasa fueron de GIBCO BRL (Geithersburg, MD). El suplemento de crecimiento de células endoteliales (ECGS), el Histopaque-1077, la polimixina B, la toxina pertúsica (PT) fueron de SIGMA Chemical Co. (St. Louis, MO); las cajas de cultivo fueron COSTAR (Minneapolis, MI). El $\text{Na}^{51}\text{CrO}_4$ y el sistema de quimioluminiscencia para detección de proteínas inmunorreactivas de western blot fue de Amersham Life Science (Buckinghamshire, England); la molécula de adhesión intercelular-1 (ICAM-1) soluble recombinante humana y los anticuerpos monoclonales (MoAb) anti-LFA-1 (anti- α L humana) y anti-VLA-4 (anti- α 4 humana) fueron de R&D Systems (Minneapolis, MN). Los MoAbs anti-PY (4G10) y anti-paxilina fueron de Upstate Biotechnology (Lake Placid, NY). Los Abs policlonales anti-JAK2, anti-STAT-3 y anti-STAT-5 fueron de Santa Cruz Biotechnology (Santa Cruz, CA). La IL-1 β se obtuvo de Genzyme Corp. (Cambridge, MA). La genisteína (GX) fue de Calbiochem (San Diego, CA). La PRL humana recombinante fue donada por Genzyme Corp (Cambridge, MA).

CULTIVOS CELULARES

a. Células endoteliales de cordón umbilical humano (HUVEC)

Para la obtención de HUVEC se empleó el método usado por Sironi y colaboradores que se describe a continuación (Sironi, et al., 1989). Se obtuvieron cordones umbilicales de partos normales, sin anestesia, con el consentimiento de la madre de la sección de toxicología del Hospital General de Zona no.1 en Querétaro. Los cordones fueron lavados con solución salina balanceada de Hank adicionada con 10 U/ml de penicilina, 10 $\mu\text{g}/\text{ml}$ de estreptomocina y 1% de fungizona. Posteriormente la vena del cordón fue lavada varias veces con esta misma solución para retirar la sangre acumulada. La vena fue cerrada por sus dos extremos e incubada 10 min a 37 °C y 5%

de CO₂ con 5-10 ml de tripsina-EDTA al 0.5%. Al final de la incubación se recuperó la suspensión de células en tripsina y se centrifugó por 10 min a 200Xg. El paquete de células fue resuspendido en medio de cultivo M-199 y sembrado en cajas de cultivo de 6 pozos forradas con FN (1 µg/cm²); estos cultivos se mantuvieron a 37 °C en una atmósfera de 95% aire y 5% de CO₂. El medio de cultivo fue suplementado con 20% de FBS, 10 U/ml de penicilina, 10 µg/ml de estreptomicina, 2 mM de L-glutamina, 25 µg/ml de heparina y 50 µg/ml de ECGS. El medio de cultivo se cambió cada tercer día.

b. Células mononucleares de sangre periférica (PBMC)

Mediante el uso del Vacutainer se obtuvo sangre periférica de donadores sanos de sexo masculino, no fumadores de 20 a 40 años de edad, que no hubieran presentado enfermedades o síntomas durante la semana previa a la extracción. La sangre obtenida se colocó en proporción 1:1 sobre una cama de Histopaque-1077 en tubos de 15 ml y se centrifugó 30 min a 400Xg con el fin de separar las células mononucleares. Las células recuperadas de la interfase del gradiente se lavaron una vez con medio de cultivo RPMI-1640 y posteriormente se colocaron en tubos de 15 ml a una densidad de 1x10⁶ células por ml con el tratamiento indicado. El medio fue suplementado con 10% FBS, 10 U/ml de penicilina, 10 µg/ml de estreptomicina y 2 mM de L-glutamina.

c. Células Jurkat

Las células Jurkat se cultivaron en medio RPMI-1640 suplementado con 10% de FBS inactivado, 10 U/ml de penicilina, 10 µg/ml de estreptomicina, 2 mM de L-glutamina, 1% de aminoácidos no esenciales y 100 mM de piruvato de sodio. El medio se cambió cada tercer día y las células se resembraron a una densidad de 2x10⁵ cels/ml.

ADHESIÓN DE PBMC A HUVEC

El método para evaluar adhesión empleado se basó en el descrito por Shimizu y colaboradores (Diag. 1)(Shimizu, et al., 1991). HUVEC (pasaje 3-6) se sembraron en platos de 96 pozos (1x10⁴ cels/pozo) cubiertos con FN (1 µg/cm²) y se cultivaron por 48 h o hasta su confluencia. Las HUVEC se activaron con 10 ng/ml de IL-1β por 4 h, se lavaron y se les colocó medio de ensayo (RPMI-1640 con 20 mM de HEPES) inmediatamente antes del inicio del experimento. Las PBMC se marcaron por 1 h a 37°C con ⁵¹Cr (35 µCi/5x10⁶) antes del tratamiento corto (5-60 min) con PRL o

después del tratamiento con PRL por 18-72 h. Las PBMC marcadas se resuspendieron en medio de ensayo, y se agregaron (5×10^4 células/pozo) por quintuplicado a los pozos con HUVEC activadas en un volumen final de 100 μ l. Las PBMC y las HUVEC se coincubaron por 1 h a 37°C con o sin los MoAbs anti-LFA-1 o anti-VLA-4 a una concentración de saturación de 10 mg/ml. En otros experimentos, las PBMC tratadas por 72 h con PRL después de ser marcadas con ^{51}Cr se incubaron en presencia o ausencia de PT (1 mM) por 2 h, se lavaron antes de la incubación con las HUVEC activadas con IL-1 β . Adicionalmente, las PBMC o células Jurkat se marcaron con ^{51}Cr y después se incubaron en presencia o ausencia de GX (10 μ M) durante 30 min, seguida de una incubación de 30 min con o sin PRL, antes de la coincubación con las HUVEC activadas. Después de la coincubación, los pozos se lavaron dos veces por inmersión en Dulbecco's-PBS (DPBS) a 37°C con 1% de BSA, sacudiendo el DPBS para remover las células no adheridas. Las HUVEC y las PBMC adheridas se lisaron con medio de ensayo con 1% de Triton-X 100, y las emisiones gamma se evaluaron en un contador de centelleo. La radiactividad pegada se expresó como porcentaje del total de radioactividad de las PBMC añadidas. La tasa de aumento es el cociente de los valores obtenidos en las muestras tratadas respecto al control no tratado.

ADHESIÓN DE PBMC A FN O ICAM-1 INMOVILIZADAS

La adhesión de PBMC a FN o ICAM-1 se evaluó de acuerdo al método reportado (Ager, 1997). Brevemente, platos para ELISA Immulon II (Dynex Technologies, Chantilly, VA) se cubrieron 1 h a temperatura ambiente con 50 μ l de FN (100 μ g/ml) o con 50 μ l de ICAM-1 (10 μ g/ml) disueltos en Dulbecco's-PBS (D-PBS). Los sitios de unión a proteína restantes de la superficie del plástico se bloquearon incubando los pozos por 1 h a temperatura ambiente con 10% de BSA en D-PBS, subsecuentemente los platos se lavaron tres veces por inmersión en D-PBS. Las PBMC tratadas o no con PRL se agregaron (5×10^4 células por pozo) a los pozos cubiertos con FN o ICAM-1 en un volumen final de 100 μ l en medio de ensayo por quintuplicado para cada condición. Las placas de ELISA se incubaron 30 min en hielo para permitir que las células se asentaran, y posteriormente 45 min a 37°C en una plataforma rotatoria (80 rpm). Después de la incubación las placas se lavaron dos veces por inmersión para remover las células no adheridas con 1%

de BSA en D-PBS precalentado a 37°C. Las células adheridas se fijaron durante 10 min con 4% de paraformaldehído en PBS y posteriormente se tiñeron con azul de toluidina al 0.1% en PBS. La adhesión se evaluó contando las células pegadas en cinco campos por pozo usando un microscopio y el software Image-Pro Plus (Media Cybernetics, Silver Springs, MD).

TRANSCRIPCIÓN REVERSA Y REACCIÓN EN CADENA DE LA POLIMERASA (RT-PCR)

El RNA total se obtuvo de las PBMC usando el método de TRIZOL (Invitrogen Corporation, Carlsbad, CA) de acuerdo al protocolo del proveedor. En breve, las células se separaron mediante centrifugación 2 min a 100Xg y se lisaron con TRIZOL (1 ml/5x10⁶ células). El lisado se incubó 5 min a temperatura ambiente y posteriormente se agregó cloroformo (0.2 ml/ml de TRIZOL). Esta suspensión se incubó a temperatura ambiente 3 min, después de lo cual se centrifugó 15 min a 12 000Xg a 4°C. La fase acuosa se separó, se mezcló con isopropanol (0.5 ml/ml de TRIZOL) y se incubó a temperatura ambiente 10 min, posteriormente se centrifugó a 12000Xg por 10 min a 4°C. El sobrenadante se descartó y el RNA se lavó con 1 ml de etanol al 75% en agua libre de RNasa. El RNA se secó y se resuspendió en agua libre de RNasa, la pureza del RNA se determinó por espectrofotometría (cociente entre $\lambda_{260}/\lambda_{280}$).

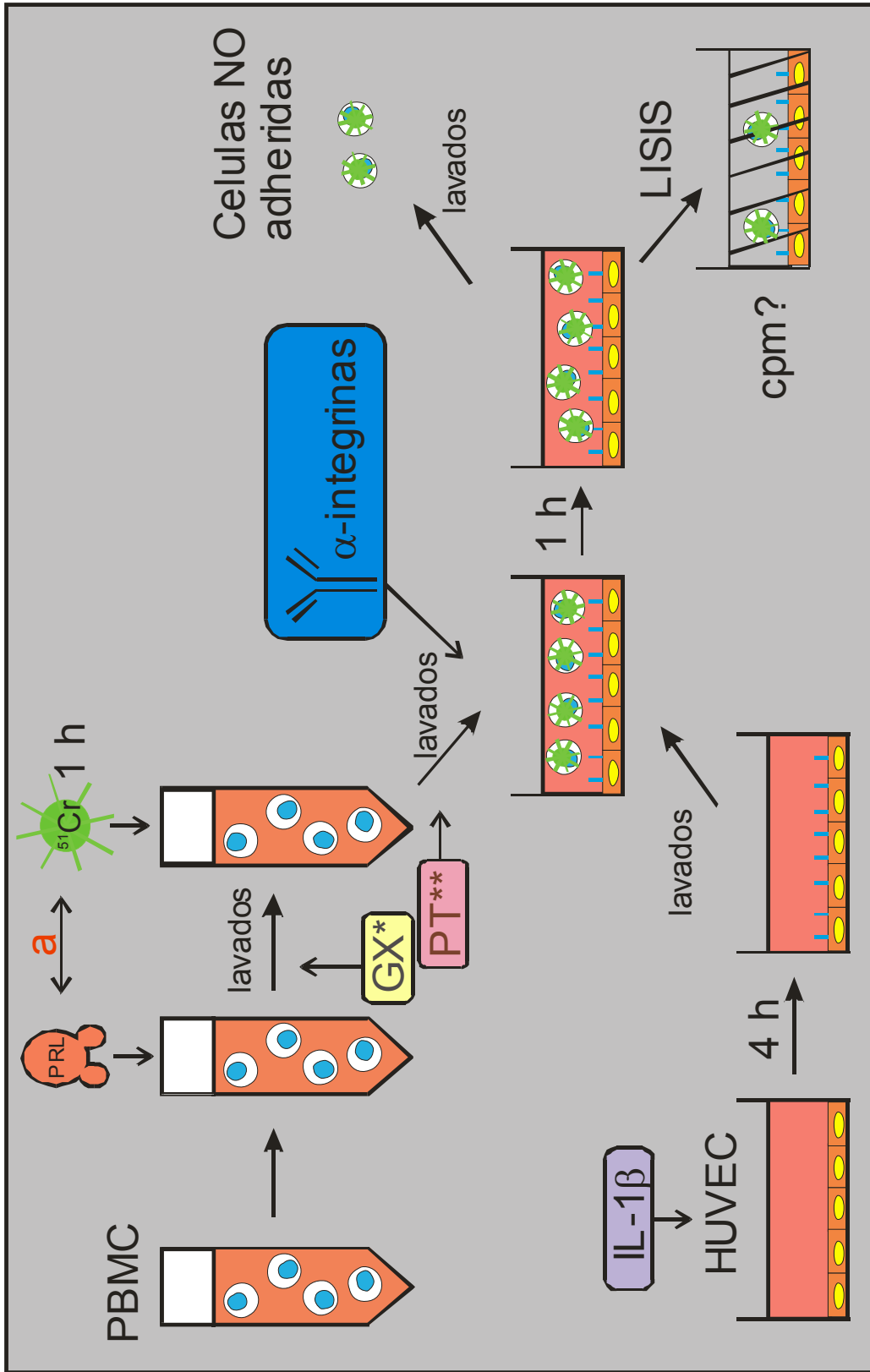


Diagrama 1. Diagrama de flujo del método empleado para evaluar adhesión de PBMC a HUVEC. a. El orden del tratamiento con PRL o el marcaje con ^{51}Cr dependió del tipo de tratamiento. Para tratamientos de hasta una hora el marcaje fue anterior al tratamiento con PRL. Para tratamientos mayores a 1 h el marcaje fue posterior al tratamiento con PRL. * En algunos experimentos las PBMC se trataron con genistéina (GX) después del marcaje y antes del tratamiento con PRL. ** En algunos experimentos las PBMC se trataron con toxina pertúsica (PT) después del tratamiento y marcaje de las PBMC.

La transcripción reversa (RT) se llevó a cabo usando 10 μg de RNA total que se mezclaron con 100 picomoles de oligo(dT). Esta mezcla se calentó a 65°C por 3 min e inmediatamente se colocó en hielo. A la mezcla se agregaron 0.1 M de DTT, 5 unidades de RNAsin, 20 mM de dNTPs y amortiguador de la enzima de retrotranscripción en un volumen total de 20 μl . Esta mezcla se llevó a 42°C en el termociclador y enseguida se añadieron 100 unidades de transcriptasa reversa de MMLV (Invitrogen Corporation, Carlsbad, CA). La reacción se llevó a cabo durante 2 h a 42°C, seguida de una incubación de 20 min a 65°C para inactivar la enzima.

Tabla I. Secuencia de oligonucleótidos, programa de PCR usado para cada uno, tamaño esperado en pb del producto y referencias.

Secuencia 5'→3'	Ciclo de T y t para PCR (desnaturalización, anidación y extensión)/no. de ciclos	pb	Referencia
CXCR1 tcc tgg gaa atg aca cag ca + aag cca aag gtg tga ggc ag -	30 seg 94°C 30 seg 60°C 30 seg 72°C 35 ciclos	64	(Patel, et al., 2001)
CXCR2 ttc cga agg acc gtc tac tca + agt ttg ctg tat tgt tgc cca tg -	30 seg 94°C 30 seg 60°C 30 seg 72°C 35 ciclos	73	(Patel, et al., 2001)
CXCR3 aac tgt ggc cga gaa agc ag + gca gtg cat cta gcc cag g -	30 seg 94°C 30 seg 60°C 30 seg 72°C 35 ciclos	68	(Patel, et al., 2001)
IP-10 agg gga gca aaa tcg atg cag t + cat cca cta aga aca tag cac ct -	30 seg 94°C 30 seg 61°C 30 seg 72°C 30 ciclos	263	NCBI Acc. No. NM_001565
ITAC ggt tac cat cgg agt tta caa ag + aag tgt gta ttt gca tga aaa aat gt -	30 seg 94°C 30 seg 61°C 30 seg 72°C 30 ciclos	295	NCBI Acc. No. NM_005409

Mig cat atg ctc tga att tat ctg agt c + aaa att att aaa acc tag ttc cac ag -	30 seg 94°C 30 seg 61°C 30 seg 72°C 30 ciclos	335	NCBI Acc. No. NM_002416
L19 cga aat cgc caa tgc caa ctc + tgc tcc atg aga atc cgc ttg -	30 seg 94°C 30 seg 60°C 30 seg 72°C	300	(Ochoa, et al., 2001)

La reacción en cadena de la polimerasa (PCR) se llevó a cabo usando 1 µl del cDNA problema, 20 µM de dNTPs, 25 picomoles/µl de los oligonucleótidos correspondientes (Tabla I) y amortiguador para Taq polimerasa en un volumen total de 48 µl. Esta mezcla se calentó a 94°C en un termociclador por 3 min, y después se llevó a 80°C. Se añadieron 5 unidades de Taq polimerasa y posteriormente se llevó a cabo el protocolo de reacción de acuerdo a lo que se indica en la Tabla I. Al final de la reacción se dejó un ciclo de 15 min a 75°C para asegurar la extensión completa de los productos de reacción. Como control de la reacción se amplificó el cDNA de la proteína ribosomal L19 (RPL19). Los productos de amplificación se analizaron en geles de agarosa. El PCR de tiempo real se llevó a cabo en reacciones que contenían: 1 µl de la alícuota de la reacción de transcripción reversa, 7.5 µl de SYBR Green PCR de Quantitec (Qiagen, Hilden, Germany) y 400 nM de oligonucleótidos, en un volumen total de 15 µl. La detección y el análisis de datos se llevaron a cabo en un LightCycler de acuerdo a las instrucciones del fabricante (Roche Molecular Biochemicals, Mannheim, Germany).

INMUNOPRECIPITACIÓN Y WESTERN BLOT (WB)

El análisis de la fosforilación de tirosinas de sustratos intracelulares se realizó siguiendo el protocolo que a continuación se describe. Células Jurkat (10×10^6) se estimularon con PRL (100-500 ng/ml) por 30 min a 37°C. Las células se recuperaron por centrifugación (5 min 100Xg) y se lisaron con 1 ml de amortiguador de lisis (50 mM Tris pH 7.4, 0.5% NP-40, 0.2% desoxicolato de sodio, 100 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 µg/ml aprotinina, 1 mM ortovanadato de sodio y 1 mM NaF). Los lisados se incubaron toda la noche con 4 µg/ml de los Abs correspondientes a 4°C. Al día siguiente se añadieron 25 µl de proteína A-sefarosa o proteína G-sefarosa y se incubaron por 1 h a 4°C. Los complejos antígeno-anticuerpo se precipitaron por centrifugación a 12000Xg por 5 min y se separaron en condiciones desnaturizantes

mediante electroforesis en gel de poliacrilamida al 12% (SDS-PAGE). Las proteínas separadas se transfirieron a una membrana de nitrocelulosa y las proteínas fosforiladas en residuos de tirosina se detectaron usando el MoAb 4G10. La incubación con dicho anticuerpo primario fue durante toda la noche a 4°C y fue seguida por una incubación de 2 h con un Ab secundario anti- IgG de ratón conjugado a HRP. Los complejos antígeno-anticuerpo se detectaron mediante quimioluminiscencia. Las mismas membranas se lavaron 5 min con NaOH 100 mM para retirar los complejos de Abs y se usaron para un segundo WB usando como Ab primario el empleado para inmunoprecipitar y así corroborar la cantidad de proteína cargada en cada carril.

ANÁLISIS DE DATOS

Todos los resultados se repitieron en tres o más experimentos independientes. Las PBMC se obtuvieron de al menos tres diferentes donadores. Los datos se expresaron como medias \pm el SEM. Las comparaciones estadísticas se realizaron mediante pruebas t de Student o ANOVA. El nivel de significancia estadística fue para valores de $p < 0.05$.

RESULTADOS

LA PRL AUMENTA LA ADHESIÓN DE PBMC A HUVEC

Las PBMC recién aisladas se trataron por diferentes tiempos con concentraciones crecientes de PRL. Posteriormente las PBMC se incubaron con HUVEC activadas pretratadas con IL-1 β . La PRL estimuló la adhesión de PBMC a HUVEC desde los 5 min de tratamiento (Tabla II). La adhesión máxima observada se alcanzó después de 1 h de tratamiento y se mantuvo durante los tratamientos más prolongados. La estimulación de la adhesión de las PBMC con concentraciones crecientes de PRL recombinante por 1 h (Fig. 8a) o 72 h (Fig. 8b) definió una curva dosis-respuesta en campana. La estimulación máxima se obtuvo con 50 ng/ml y produjo un aumento de la adhesión del 60% y 67% para los tratamientos con PRL de 1 h y 72 h, respectivamente.

LAS INTEGRINAS VLA-4 Y LFA-1 MEDIAN EL EFECTO DE LA PRL SOBRE LA ADHESIÓN CELULAR

Para investigar si las integrinas VLA-4 y LFA-1 están involucradas en el efecto estimulador de PRL sobre la adhesión, se hicieron experimentos de adhesión de PBMC a HUVEC en presencia de MoAbs anti-LFA-1 (anti- α L) y anti-VLA-4 (anti- α 4) que bloquean la adhesión mediada por estas integrinas. En estos experimentos se encontró que la adhesión estimulada después de 72 h de incubación con 50 ng/ml de PRL disminuyó en un 60% con los anticuerpos anti-VLA-4, mientras que los MoAbs anti-LFA-1 tuvieron un efecto pequeño no significativo. Sin embargo cuando estos MoAb se usaron en combinación, la adhesión estimulada por PRL disminuyó en más del 80%. El efecto de los Abs anti-integrinas fue específico, ya que la presencia de un Ab control del mismo isotipo (IgG1) no tuvo efecto sobre la adhesión inducida por PRL (Fig. 9). Estos resultados indican que las integrinas VLA-4 y LFA-1 están involucradas en el efecto estimulador de la adhesión inducido por PRL.

Considerando estos resultados, se analizó la adhesión de PBMC a sustratos solubles de las integrinas VLA-4 y LFA-1 inmovilizados en plástico. El tratamiento por

72 horas con diferentes concentraciones de PRL también aumentó la adhesión de PBMC a ICAM-1, ligando de LFA-1 (Fig. 10A) y a FN, ligando de VLA-4. En estos experimentos encontramos que la PRL aumentó la adhesión de las PBMC a ICAM-1 hasta en un 50% en comparación con la adhesión basal, mientras que el aumento de adhesión a FN fue de 40% (Fig. 10B). De manera similar a los experimentos descritos de adhesión a HUVEC, el efecto de concentraciones crecientes de PRL produjo una curva dosis-respuesta en forma de campana.

Dado que sabemos que los LPS de bacterias Gram-negativas pueden estimular la adhesión de leucocitos mediada por integrinas, se analizó la posibilidad de que el efecto observado involucrara a este contaminante ubicuo. Para ello se determinó la adhesión de PBMC a FN inmovilizada en plástico en presencia del antibiótico inhibidor de LPS polimixina-B. En estos experimentos el aumento de adhesión inducido por PRL no fue bloqueado por el antibiótico (Fig. 11), lo que indica que el efecto observado no se debe a la presencia de LPS en las preparaciones de PRL.

LA PRL INDUCE LA EXPRESIÓN DEL RECEPTOR A QUIMIOCINAS CXCR3 EN PBMC

Con la finalidad de estudiar la posible contribución de las quimiocinas en la adhesión de PBMC inducida por PRL, se investigó el efecto de esta hormona sobre la expresión de los receptores a quimiocinas CXCR1, CXCR2 y CXCR3, cuyos ligandos selectivos son las quimiocinas que comparten un arreglo CXC de sus dos primeros residuos de cisteína. El RNA total de PBMC tratadas o no con PRL (50 ng/ml) por 72 h fue analizado por RT-PCR. Los productos de amplificación obtenidos tuvieron los tamaños esperados (Tabla I) para CXCR3 (68 bp), CXCR2 (73 bp) y CXCR1 (64 bp). La expresión de CXCR3 apenas se detectó en PBMC no tratadas, mientras que el tratamiento con PRL aumentó marcadamente su expresión (Fig. 12A). Este hallazgo se corroboró mediante PCR en tiempo real, y se encontró que la PRL aumentó 2.5 veces la expresión del mRNA de CXCR3 en PBMC (Fig. 12B). De manera contrastante, la expresión del mRNA de CXCR2 y CXCR1 fue similar en presencia o ausencia del tratamiento con PRL (Fig. 12A). Adicionalmente, cuando se investigó por RT-PCR la

expresión de las quimiocinas CXC de respuesta a IFN- γ (IP-10, I-TAC y MIG), se obtuvieron productos de amplificación de tamaño esperado para IP-10 (263 bp), I-TAC (295 bp) y MIG (335 bp) (Fig. 12C), lo que sugiere que las HUVEC expresan los tres ligandos conocidos de CXCR3. Finalmente, el tratamiento con toxina pertúsica, un inhibidor de proteínas G, bloqueó la estimulación inducida por PRL de la adhesión de PBMC a HUVEC (Fig. 12D), sugiriendo la contribución de receptores acoplados a proteínas G, posiblemente de CXCR3, en el aumento de adhesión inducido por PRL.

LA PRL PROMUEVE LA ADHESIÓN CELULAR A TRAVÉS DE LA ACTIVACIÓN DE TIROSINA CINASAS

En este trabajo también se investigó si las vías de fosforilación de tirosinas pueden contribuir al efecto de PRL sobre adhesión, usando genisteína (GX), un inhibidor típico de tirosina cinasas. En estos experimentos, las PBMC o las células Jurkat fueron incubadas con GX por 30 min y después con o sin PRL por 30 min más. Inmediatamente, las células fueron incubadas por 1 h con HUVEC activadas con IL-1 β . En consistencia con los hallazgos previos, el tratamiento de las células con PRL estimuló la adhesión de PBMC o de las células Jurkat a HUVEC con una curva dosis-respuesta en forma de campana. La GX por su parte, bloqueó el efecto de PRL en PBMC y células Jurkat (Fig. 13A y B). Posteriormente, se investigó el efecto de PRL sobre la fosforilación de tirosinas de las proteínas JAK2, STAT3, STAT5 y paxilina. Para esto, las células Jurkat se incubaron por 30 min con diferentes concentraciones de PRL, se lisaron y estos lisados se sometieron a inmunoprecipitación y WB. El análisis por WB con anticuerpos antifosfotirosina (4G10), mostró que la PRL indujo la fosforilación de tirosinas de JAK2, STAT3, STAT5 y paxilina (Fig. 14). Cuando esta reacción se eliminó, y las membranas fueron reveladas con Abs que reconocen a las proteínas totales respectivas, se corroboró que la misma cantidad de cada una de las proteínas se había cargado en cada carril, por lo que el aumento en fosforilación era real. El efecto de la PRL sobre la fosforilación de tirosinas de paxilina se verificó inmunoprecipitando directamente con el Ab anti-fosfotirosina y revelando el blot con el Ab anti-paxilina. Este acercamiento también mostró que el tratamiento con PRL aumenta la cantidad de fosforilación de tirosinas de paxilina (Fig. 14).

Tabla II. Efecto de PRL sobre la adhesión de PBMC a HUVEC

	Tiempo						
	5 min	15 min	30 min	1 h	6 h	18 h	72 h
Control	20.6±0.67	21.5±0.95	19.7±1.2	17.2±0.81	14.3±0.62	12.5±0.47	9.2±0.76
PRL (50 ng/ml)	27.5±0.82*	30.3±0.86*	27.3±0.62*	28.2±1.33*	22.5±1.5*	19.4±1.71*	15.4±1.24*
Tasa de aumento	1.33	1.41	1.39	1.64	1.57	1.55	1.67

Los valores mostrados son porcentajes de adhesión de la radioactividad unida respecto a la radioactividad total de las PBMC sembradas. Las tasas de aumento representan el cociente de los valores obtenidos en las muestras tratadas con PRL respecto al control no tratado. Las PBMC se trataron con PRL (50 ng/ml) a 37°C por el intervalo de tiempo indicado. Las PBMC se marcaron con ^{51}Cr y se incubaron con células HUVE tratadas 4 h con IL-1 β . Las células no adheridas se lavaron y se evaluó la radioactividad unida y total por pozo. * $p < 0.05$ vs PBMC no tratadas con PRL.

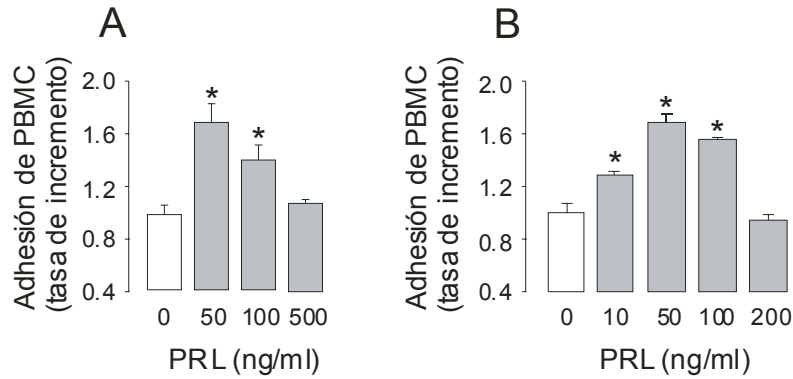


Figura 8. Efecto de PRL sobre la adhesión de PBMC a HUVEC. Adhesión de PBMC a HUVEC después de un tratamiento de 1 h (A) o 72 h (B) con PRL. Los resultados representan la media \pm SEM de quintuplicados. * $p < 0.05$ vs. el control no tratado. Se muestra un experimento representativo de al menos tres experimentos independientes.

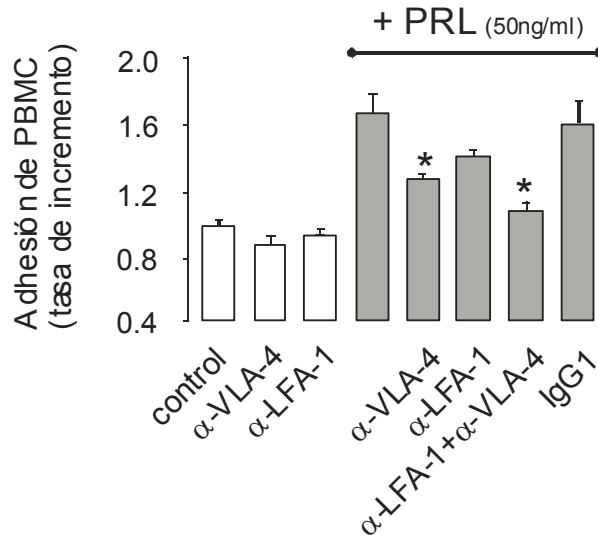


Figura 9. Bloqueo del efecto de PRL sobre la adhesión de PBMC a HUVEC con MoAb anti-integrinas LFA-1 y VLA-4. Efecto de los MoAb anti-LFA-1 y/o anti-VLA-4 sobre la adhesión de PBMC a HUVEC inducida por 72 h de tratamiento con PRL. Los resultados representan la media \pm SEM de quintuplicados. * $p < 0.05$ vs. PBMC tratadas con 50 ng/ml PRL. Se muestra un experimento representativo de cuatro experimentos independientes.

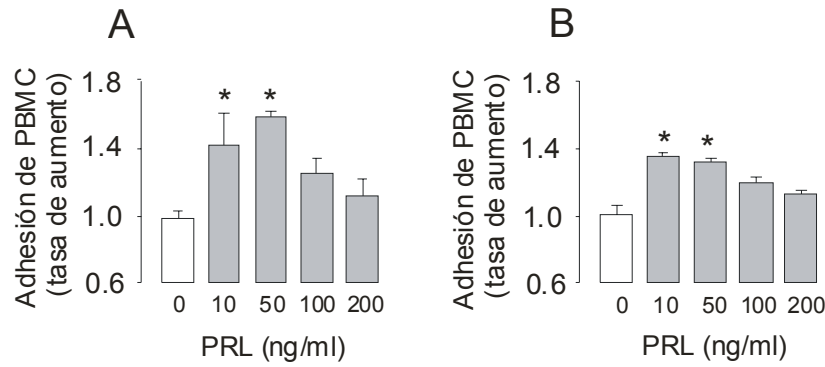


Figura 10. Efecto de PRL sobre la adhesión de PBMC a ICAM-1 (A) y FN (B). Las PBMC fueron tratadas por 72 h con PRL y posteriormente incubadas en platos cubiertos con ICAM-1 o FN como se describe en métodos. Los resultados representan la media \pm SEM de quintuplicados. * $p < 0.05$ vs. el control no tratado. Se muestra un experimento representativo de al menos tres experimentos independientes.

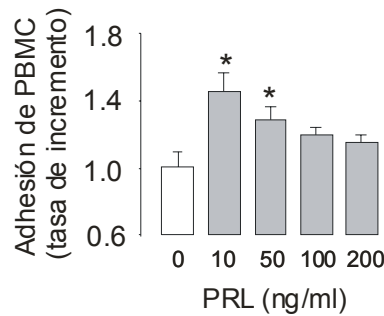


Figura 11. Efecto de PRL sobre la adhesión de PBMC a FN en presencia de polimixina B. Las PBMC fueron tratadas por 72 h con PRL en presencia de polimixina-B y su adhesión a FN se evaluó como se describe en materiales y métodos. Los resultados representan la media \pm SEM de quintuplicados. * $p < 0.05$ vs PBMC no tratadas con PRL. Se muestra un experimento representativo de cuatro experimentos independientes.

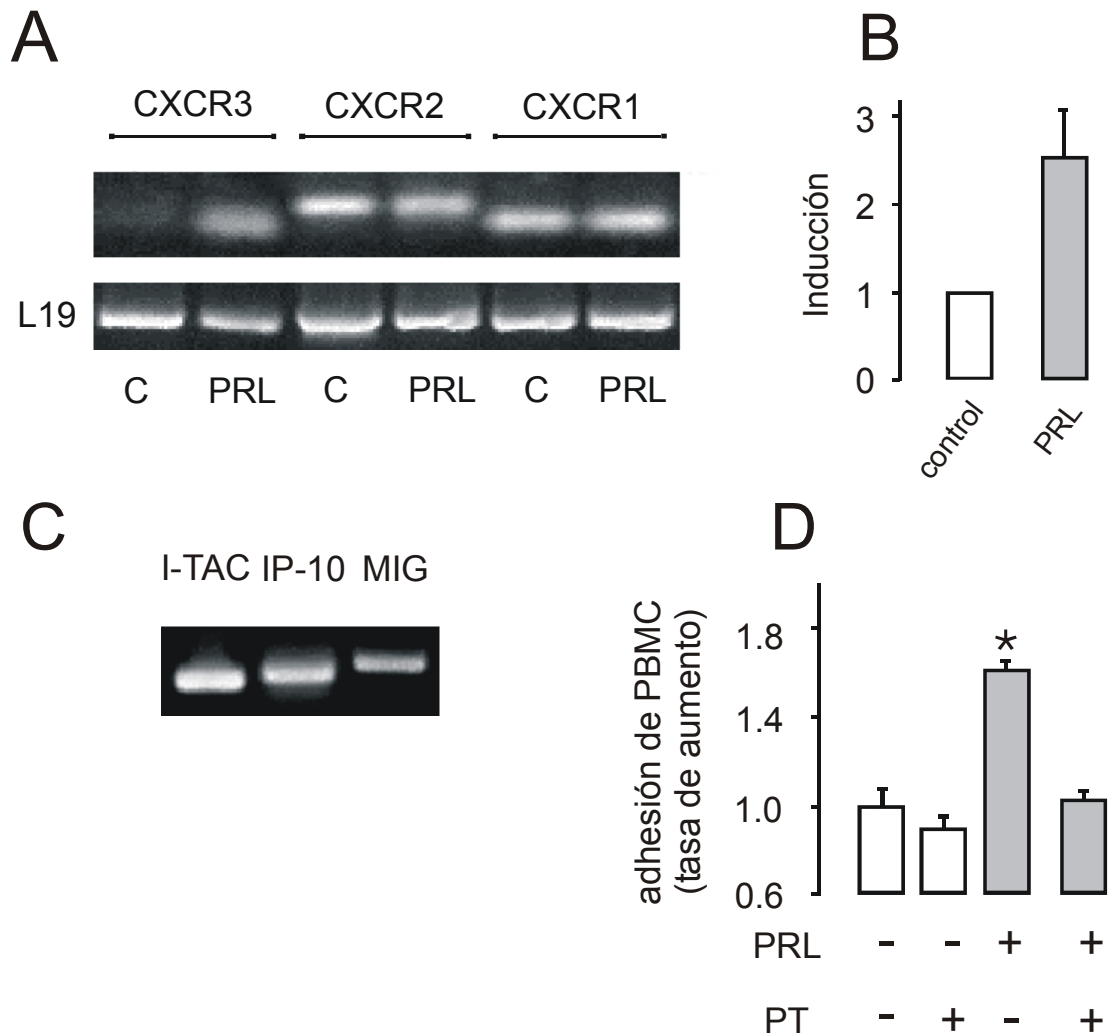


Figura 12. Efecto de PRL sobre la expresión de receptores a quimiocinas en PBMC. **A.** RT-PCR del mRNA de los receptores CXCR1, CXCR2 y CXCR3 en PBMC tratadas con 50 ng/ml de PRL por 72 h. La expresión de RPL19 se muestra como control **B.** Cuantificación de la expresión de CXCR3 mediante PCR tiempo real. **C.** RT-PCR del mRNA de los ligandos de CXCR3 IP-10, I-TAC y MIG en HUVEC **D.** Efecto inhibitorio de la toxina pertusica (PT) sobre la adhesión inducida por PRL de PBMC a HUVEC. Las PBMC tratadas o no con PRL por 72 h se incubaron en presencia o ausencia de PT (1 mM) por 2 h antes de la incubación con HUVEC. Los resultados representan la media \pm SEM de triplicados. * $p < 0.05$ vs PBMC no tratadas con PRL. Se muestran experimentos representativos de tres experimentos independientes, con excepción de (B) en el que se muestra la media de tres experimentos independientes.

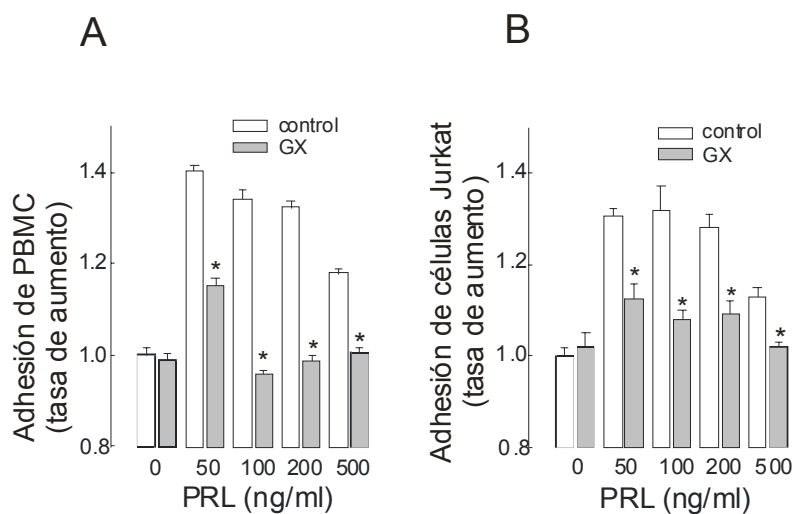


Figura 13. Bloqueo por GX del efecto de PRL sobre la adhesión de PBMC (A) y células Jurkat (B) a HUVEC. Las PBMC o células Jurkat fueron preincubadas por 30 min con genisteína (GX, 10 μ M) y posteriormente tratadas con o sin PRL por 30 min. La adhesión de las células se evaluó como se describe en materiales y métodos. Los resultados representan la media \pm SEM de quintuplicados. * $p < 0.05$ vs la adhesión de células no tratadas con GX. Se muestra un experimento representativo de tres experimentos independientes.

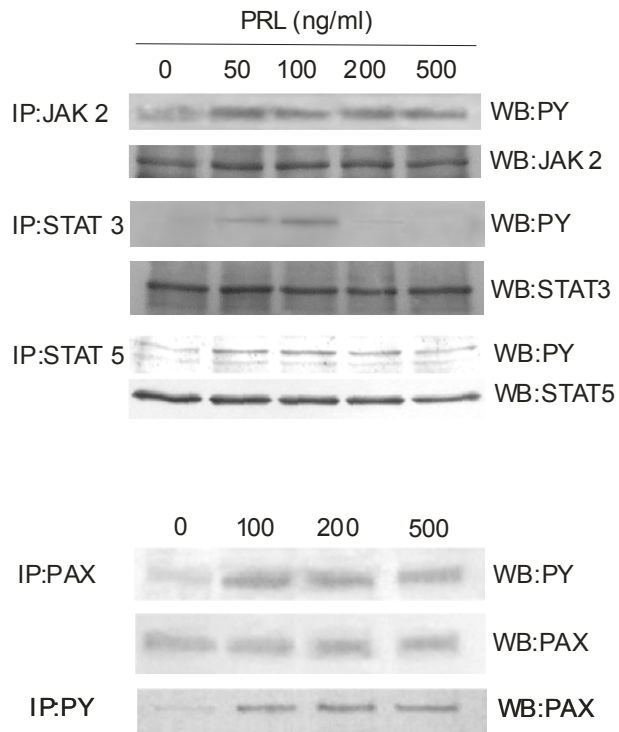


Figura 14. Inducción por PRL de la fosforilación de tirosinas. La PRL induce la fosforilación de tirosinas de JAK2, STAT3, STAT5 y paxilina (PAX) en células Jurkat. Las células Jurkat se trataron con las concentraciones indicadas de PRL por 30 min y los lisados celulares se inmunoprecipitaron (IP) con el Ab indicado. Enseguida, se llevaron a cabo análisis de WB y revelados secuenciales con los Abs indicados como se describe en materiales y métodos.

DISCUSIÓN

Múltiples evidencias indican que la PRL participa en diversos aspectos de la respuesta inmune, incluyendo el proceso inflamatorio (Yu-Lee, 2002). No obstante, modelos de animales genéticamente modificados para PRL y el PRLR han puesto en duda el papel de esta hormona, ya que en estos modelos experimentales no se modifica el desarrollo de la respuesta inmune humoral y celular (Bole-Feysot, et al., 1998; Foster, et al., 2000). Si bien es posible que la PRL no sea indispensable para el desarrollo normal de la respuesta inmune, se ha propuesto que esta hormona puede participar en mantener la homeostasis del organismo en situaciones de estrés bajo trauma, infección e inflamación (Dorshkind and Horseman, 2001; Yu-Lee, 2002). Cabe señalar, que este tipo de contradicciones se han encontrado para otro tipo de moléculas fundamentales del sistema inmunológico como por ejemplo IL-2. En experimentos llevados a cabo en el ratón KO de esta citosina no se encontraron deficiencias en respuestas de células T, B y NK reportadas en otros modelos (Swain, 1991; Kundig, et al., 1993).

Los experimentos aquí reportados demuestran que la PRL estimula la adhesión mediada por integrinas de leucocitos a células endoteliales. Encontramos que concentraciones de PRL, similares a las reportadas en humanos en condiciones normales (10 ng/ml) y con hiperprolactinemia (>20 ng/ml), elevan la adhesión de PBMC a HUVEC (Tabla II, Fig. 8) (Neidhart, et al., 1999; Cruz, et al., 2001). El aumento máximo observado (70%) fue menor al reportado con IL-2 (200%), citocina que se sabe aumenta la adhesión de leucocitos a endotelio (Pankonin, et al., 1992; Nielsen, et al., 1996). No obstante, el aumento inducido por PRL fue mayor al efecto máximo reportado de GH (30%) sobre la adhesión de neutrófilos, hormona emparentada con PRL con acciones sobre el sistema inmune (Ryu, et al., 2000).

En este trabajo también encontramos que las integrinas LFA-1 y VLA-4 median la adhesión inducida por PRL. Los resultados de estos experimentos sugieren que VLA-4 tiene una mayor contribución que LFA-1 en la adhesión inducida por PRL, aunque se necesitan más experimentos para concluir en este sentido. No obstante, podría ocurrir que la participación de LFA-1 no fuera tan evidente debido a mecanismos de regulación cruzados que han sido reportados para ambas integrinas, y que involucran cambios

funcionales de estas moléculas ocasionados por la unión a su ligando (Porter and Hogg, 1997; Chan, et al., 2000; May, et al., 2000). En este sentido es posible que la unión mediada por LFA-1 sea estimulada por la unión de VLA-4 a su ligando, mecanismo que ya ha sido reportado en linfocitos T (Chan, et al., 2000; May, et al., 2000). Por otro lado, dado que el Ab anti-VLA-4 está dirigido contra la subunidad α de esta integrina, es posible que la integrina LPAM-1 ($\alpha 4\beta 7$) también esté involucrada en el efecto de PRL. Sin embargo, debido a que la distribución de esta integrina está restringida sólo a algunas subpoblaciones de linfocitos T, su participación puede ser limitada.

La regulación de las integrinas VLA-4 y LFA-1 en la adhesión inducida por PRL se verificó evaluando la adhesión de las PBMC a sustratos solubles de estas integrinas (FN e ICAM-1). En consistencia con los resultados antes descritos, encontramos que la PRL también aumenta la adhesión de PBMC a FN e ICAM-1 inmovilizadas en plástico. Es importante hacer notar que la inducción de la adhesión por PRL de PBMC a HUVEC, FN e ICAM-1 generó curvas dosis-respuesta en forma de campana, lo que sugiere un efecto específico de PRL sobre el PRLR clonado y caracterizado en monocitos y linfocitos, tipos celulares mayormente representados en las PBMC (Clevenger, et al., 1998). La forma de la curva sugiere la especificidad del efecto por PRL debido a que, de acuerdo al modelo de activación del PRLR, se requiere que una molécula de PRL dimerice a dos PRLR, para iniciar su cascada de señales, lo que ocasiona que a concentraciones altas de hormona no haya dimerización y por lo tanto efecto (Fuh, et al., 1993; Clevenger, et al., 1998).

Asimismo nuestros experimentos descartaron la posibilidad de que LPS pudiera participar en el aumento de adhesión observado con PRL. Se sabe que estos componentes de la membrana externa de bacterias Gram-negativas son capaces de estimular la adhesión de leucocitos a endotelio mediante la inducción de mediadores proinflamatorios como IL-1, IL-6, IL-8 o TNF- α (Davenpeck, et al., 1998; Chow, et al., 1999). Sin embargo, cuando se probó el efecto de PRL sobre la adhesión de PBMC a FN en presencia de polimixina B, encontramos que este antibiótico no bloqueó el aumento de adhesión. Más aún, en estudios no descritos en este trabajo, hemos encontrado que la concentración de LPS en las preparaciones de PRL utilizadas en este estudio es 300 veces menor que la necesaria para inducir un aumento de adhesión

cercano al 40%. Estas observaciones sugieren que el efecto observado de PRL sobre la adhesión de PBMC no se debe a LPS. La interferencia de este contaminante en el efecto de otras prolactinas recombinantes ya ha sido reportada y evaluada por otros grupos, por lo que descartar esta posibilidad era conveniente en este trabajo (Galfione, et al., 2003).

El mecanismo mediante el cual la PRL aumenta la adhesión de PBMC puede estar asociado a la participación de quimiocinas. Las quimiocinas constituyen una familia de citocinas quimiotácticas que actúan uniéndose a sus receptores que pertenecen a la familia de receptores acoplados a proteínas G. Las características funcionales de estas moléculas sugieren que su regulación y expresión así como la de sus receptores, juega un papel importante en la extravasación de leucocitos (Ebnet, et al., 1996; Baggiolini, et al., 1997; Rot and von Andrian, 2004). En este contexto realizamos experimentos para examinar el efecto de PRL sobre tres diferentes receptores a quimiocinas: CXCR1, CXCR2 y CXCR3. En estos experimentos encontramos que la PRL induce la expresión de CXCR3. Se ha reportado que este receptor se expresa principalmente en células T activadas con IL-2, aunque se ha encontrado también en linfocitos B, monocitos y neutrófilos (Piali, et al., 1998). Se sabe que la activación de CXCR3 por sus ligandos aumenta la adhesión de linfocitos T mediada por las integrinas LFA-1 y VLA-4 (Piali, et al., 1998; Patel, et al., 2001). Los ligandos de CXCR3 son las quimiocinas de respuesta a γ -IFN: IP-10, ITAC y MIG (Murdoch and Finn, 2000).

Para que el aumento de expresión de CXCR3 en las PBMC tenga implicaciones funcionales en la mediación del efecto de PRL, se requiere que al menos uno de sus ligandos esté presente durante el ensayo y active al receptor. Esto es posible ya que encontramos que las HUVEC expresan los tres ligandos conocidos de CXCR3. Más aún, se sabe que las quimiocinas pueden concentrarse en el microambiente próximo del endotelio, ya que se ha reportado que las quimiocinas pueden asociarse a glicosaminoglicanos de la ECM del endotelio y así favorecer la interacción con su receptor (Ebnet, et al., 1996; Baggiolini, et al., 1997; Rot and von Andrian, 2004). Adicionalmente, encontramos que el tratamiento de las PBMC con PT bloquea el aumento de adhesión inducido por PRL, lo que indica la participación de receptores acoplados a proteínas G. Estos resultados sugieren que el aumento de expresión de CXCR3 en las PBMC puede estar involucrado en el aumento de adhesión inducido por

PRL. En consistencia con esta posibilidad, se ha encontrado un alto número de células CXCR3 positivas en lesiones internas de pacientes con RA, enfermedad en la que se han reportado altos niveles de PRL en suero y líquido sinovial (Qin, et al., 1998; Sorensen, et al., 1999).

En este trabajo encontramos también que la PRL no sólo estimula la adhesión de leucocitos a endotelio después de una exposición de horas, sino incluso después de tratamientos breves. Si bien la regulación de la expresión de genes como quimiocinas y sus receptores es uno de los mecanismos mediante el cual PRL puede estimular la adhesión de leucocitos a endotelio, los resultados obtenidos con tratamientos cortos de PRL sugieren que existen otros mecanismos celulares involucrados en este efecto. Un mecanismo posible es la activación de cinasas que participan en la formación de placas focales de adhesión. Para investigar si la activación de tirosina cinasas participa en la inducción de la adhesión por PRL, determinamos el efecto de GX sobre la adhesión estimulada por PRL de PBMC y células Jurkat a HUVEC. Este inhibidor de tirosina cinasas redujo de manera significativa el efecto de PRL sobre la adhesión de ambos tipos celulares al endotelio vascular, señalando la participación de tirosina cinasas en el efecto de PRL. En vista de estos resultados, investigamos el efecto de la PRL sobre la fosforilación de tirosinas de JAK2, STAT3, STAT5 y paxilina en la línea celular Jurkat. Esta línea celular es derivada de una leucemia humana de células T, expresa el PRLR y ha sido empleada por otros grupos para evaluar el efecto de PRL sobre la biología celular de los leucocitos (LaGree, et al., 1988; Pellegrini, et al., 1992; Matera, et al., 1997). En estos experimentos encontramos que al igual que en otros tipos celulares (Bole-Feysot, et al., 1998), la PRL estimula la fosforilación de tirosinas de JAK2, STAT3 y STAT5, y de paxilina (Bole-Feysot, et al., 1998). La PRL estimula la activación de la fosforilación de tirosinas de paxilina vía la activación de JAK/STAT en células cancerosas de mama en respuesta a PRL (Canbay, et al., 1997) y la GH hace lo mismo en neutrófilos (Ryu, et al., 2000; Zhang, et al., 2001). La paxilina es una proteína adaptadora que se une a las colas citoplásmicas de las integrinas y que participa en la coordinación temporal y espacial del armado de las AF (Nakamura, et al., 2000; Turner, 2000). Ahí, provee una plataforma para la integración y procesamiento de señales relacionadas con la adhesión y factores de crecimiento (Nakamura, et al., 2000; Turner, 2000). Se ha reportado que la activación de paxilina estimula la migración dependiente de LFA-1 promoviendo la activación de Pyk2 y FAK (Rose, et al., 2003). En conjunto,

estos resultados señalan que la activación de tirosina cinasas, comúnmente asociadas a la cascada de señales del PRLR, median la inducción de la adhesión por PRL, lo que a su vez provoca la activación de paxilina, permitiendo así la adhesión celular. Cabe señalar que existen otras moléculas candidatas a participar en el efecto de PRL sobre la adhesión mediada por integrinas. Se sabe que la afinidad de las integrinas puede ser regulada intracelularmente por cinasas como ZAP-70, PI3K, Ras, Raf, PLC, FAK, PKC, y cinasas de la familia src, moléculas que también están involucradas en la transducción de señales iniciada por la activación del PRLR (Canbay, et al., 1997; Bole-Feysot, et al., 1998; Hughes and Pfaff, 1998; Montgomery, et al., 1998; Epler, et al., 2000). No obstante se necesitan más experimentos para dilucidar con precisión la participación de estas moléculas en el aumento de adhesión inducido por PRL. Adicionalmente, existen otros procesos intracelulares que son activados por la cascada de señales del PRLR y que pueden estar relacionados con el aumento de adhesión. Se sabe que PRL aumenta la liberación de Ca^{++} intracelular, y se ha reportado que el aumento intracelular de este catión puede regular la afinidad de integrinas a través de la acción de calreticulina (Hughes and Pfaff, 1998).

En resumen los resultados obtenidos de este trabajo demuestran que el tratamiento con PRL aumenta la adhesión mediada por integrinas de leucocitos a endotelio. Además, hemos encontrado evidencias de que este efecto de PRL puede estar regulado a través de al menos dos mecanismos diferentes: la activación de cascadas intracelulares mediadas por tirosina cinasas y la regulación de la expresión de receptores a quimiocinas. El resumen de estos resultados se esquematiza en la Fig. 15.

Considerando estos resultados y reportes previos que involucran a PRL con la respuesta inflamatoria es posible que esta hormona participe de manera local y/o sistémica en la extravasación de leucocitos (Yu-Lee, 2002). La participación local de PRL en la adhesión de leucocitos es posible ya que se ha encontrado que diferentes subpoblaciones de leucocitos y células endoteliales producen PRL (Wu, et al., 1996; Bole-Feysot, et al., 1998; Clapp, et al., 1998; McMurray, 2001a). Además, se ha reportado la expresión de PRL en tejidos linfoides como el bazo o los nódulos linfáticos, en los cuales la extravasación de leucocitos es un evento de alta ocurrencia (Wu, et al., 1996). Por otro lado, la acción sistémica de PRL sobre la adhesión de leucocitos es apoyada por el hecho de que pacientes con enfermedades autoinmunes

como SLE o RA presentan hiperprolactinemia, lo que podría estar relacionado con los patrones alterados de migración de leucocitos que presentan estos pacientes (McMurray, 2001b). Más aún, la BCR (inhibidor de la producción de PRL hipofisiaria) ha sido empleada como terapia en pacientes con RA y SLE con resultados positivos (McMurray, 2001b; Yang, et al., 2003).

Este trabajo es el primero que reporta la acción directa de PRL sobre la adhesión mediada por integrinas de leucocitos a endotelio, y esto apoya la participación de PRL en procesos de inflamación e infección, en los cuales el tráfico de leucocitos es crucial (Meli, et al., 1993; Tangbanluekal and Robinette, 1993; Zellweger, et al., 1996b; Yu-Lee, 2002). En este sentido, los cambios en las concentraciones local y circulante de PRL podrían estimular la función inmune regulando la adhesión de leucocitos a endotelio favoreciendo así su migración. Es bien sabido que existe una amplia gama de agentes estresantes que estimulan la liberación de PRL en la adenohipófisis, y se ha propuesto que la PRL podría contrarrestar los efectos negativos de los esteroides y otros agentes supresores liberados también en momentos de estrés (Kelley, 1991; Ben-Jonathan, et al., 1996; Van de Kar and Blair, 1999; Dorshkind and Horseman, 2001; Krishnan, et al., 2003).

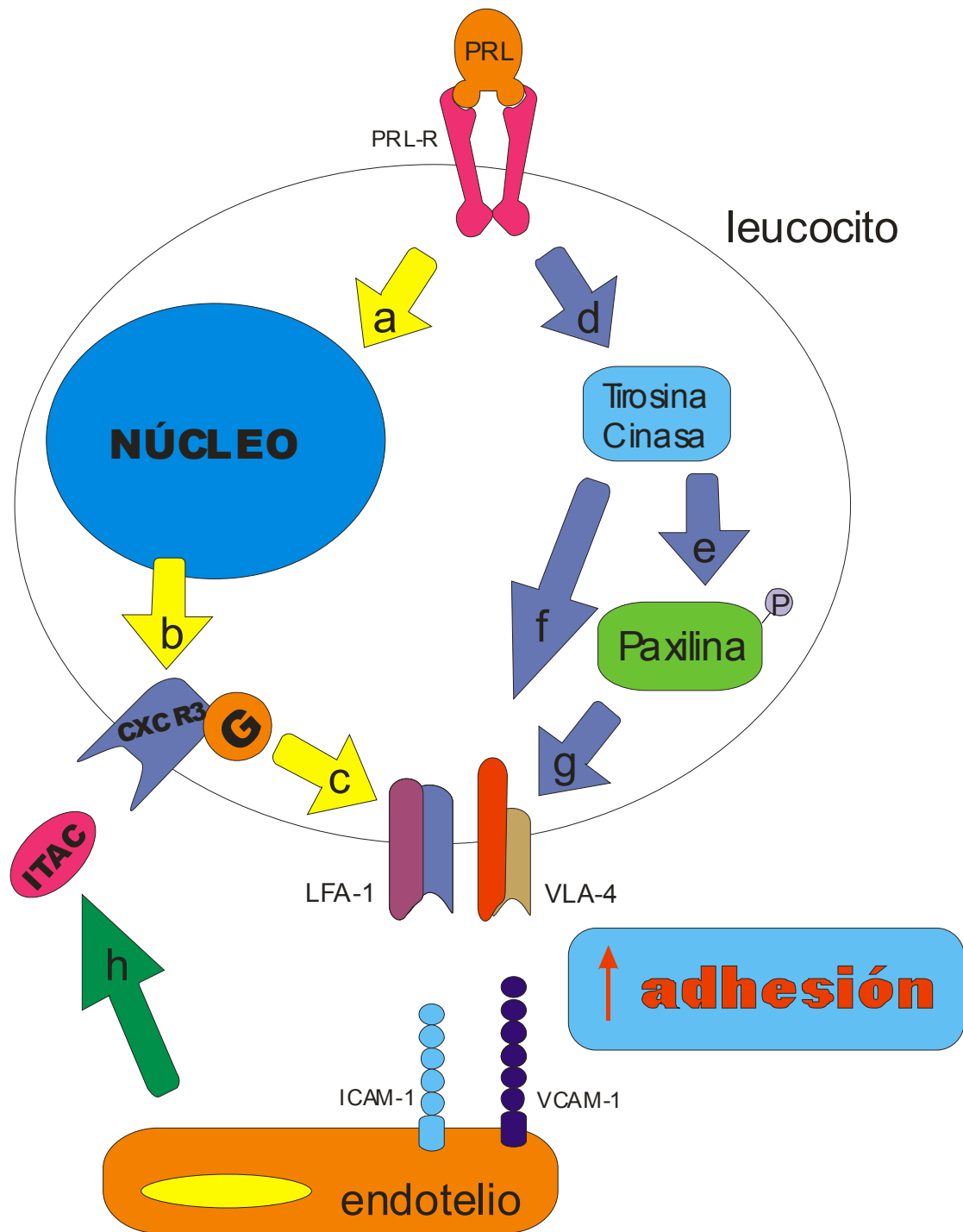


Figura 15. Esquema de los mecanismos propuestos que intervienen en el efecto de PRL sobre la adhesión de leucocitos. El tratamiento con PRL inicia cascadas de señales (a) que aumentan la expresión de genes como CXCR3 (b), el cual al ser activado por quimiocinas como ITAC producidas por el endotelio (h) estimula a través de proteínas G y otros segundos mensajeros (c) la adhesión mediada por las integrinas LFA-1 y VLA-4. Por otro lado, el tratamiento con PRL inicia cascadas de señales (d) que involucran a JAK, STATs y

probablemente otras tirosinas cinasas, las que a través de paxilina (**e**) u otras vías (**f**) estimulan la adhesión mediada por las integrinas LFA-1 y VLA-4.

La participación de PRL en la regulación de la respuesta inmune ha sido sugerida por diversos autores, debido a la correlación que existe entre la hiperprolactinemia y el desarrollo de enfermedades autoinmunes como SLE, esclerosis múltiple y RA (Velkeniers, et al., 1998; Cruz, et al., 2001; McMurray, 2001b; Pacilio, et al., 2001). Esta hipótesis ha tomado renovado interés gracias a lo que se conoce como el hueco de género (gender gap), que refleja la mayor incidencia de enfermedades autoinmunes en mujeres que en hombres (Velkeniers, et al., 1998; McMurray, 2001a; Whitacre, 2001). En este sentido, es bien conocido que la PRL es una hormona cuyos niveles en suero son mayores en mujeres que en hombres (Sinha, 1995; Ben-Jonathan, et al., 1996; Freeman, et al., 2000). Existen, además, evidencias experimentales que apoyan la participación de PRL en el desarrollo de estas enfermedades. En pacientes con RA se ha reportado una elevada expresión y función de la integrina VLA-4 de linfocitos T (Laffon, et al., 1991). Asimismo, se ha reportado que la PRL producida localmente por linfocitos T infiltrados en lesiones de enfermos con RA regula la función de las células sinoviales, aumentando la producción de citocinas proinflamatorias y de metaloproteinasas, lo que actúa en detrimento de las lesiones (Nagafuchi, et al., 1999). Nuestros resultados apuntalan la posibilidad de que PRL esté involucrada en el desarrollo de enfermedades autoinmunes, debido a la importancia que tiene la extravasación de leucocitos en el desarrollo de la respuesta inflamatoria. Sin embargo, es importante considerar que este tipo de enfermedades resulta de un desbalance de la homeostasis del organismo, lo que implica necesariamente que existen otros factores como la base génica o los factores ambientales que al integrarse ayudan al desarrollo de estas enfermedades (Whitacre, 2001). De este modo, es importante continuar con el estudio de los factores que ayudan al desarrollo de las enfermedades autoinmunes, así como con el análisis del papel de PRL, para conocer los alcances y limitaciones de la participación de esta hormona.

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APENDICE 1

Prolactin stimulates integrin-mediated adhesion of circulating mononuclear cells to endothelial cells

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Attachment of leukocytes to endothelial cells is an essential step for the extravasation and recruitment of cells at sites of inflammation. The pituitary hormone prolactin (PRL) is involved in the inflammatory process. Here, we show that treatment with PRL of human peripheral blood mononuclear cells (PBMC) stimulates their adhesion to human umbilical vein endothelial cells (HUVEC) activated by interleukin-1 β . Stimulation of adhesion by PRL is mediated via integrins leukocyte functional antigen-1 (LFA-1) and very late antigen-4 (VLA-4), because immunoneutralization of both integrins prevents PRL action. Also, PRL promotes the adhesion of PBMC to immobilized intercellular adhesion molecule-1 and fibronectin, ligands for LFA-1 and VLA-4, respectively. Stimulation of integrin-mediated cell adhesion by PRL may involve the activation of chemokine receptors, because PRL upregulates the expression of the G-protein-coupled chemokine receptor CXCR3 in PBMC and pertussis toxin, a specific G-protein inhibitor, blocks PRL stimulation of PBMC adhesion to HUVEC. In addition, PRL stimulates tyrosine phosphorylation pathways leading to leukocyte adhesion. PRL triggered the tyrosine phosphorylation of Janus kinase-2 of signal transducer and activator of transcription-3 and 5, and of the focal adhesion protein paxillin. Furthermore, genistein, a tyrosine kinase inhibitor, blocked PRL-stimulated adhesion of PBMC and Jurkat T-cells to HUVEC. These results suggest that PRL promotes integrin-mediated leukocyte adhesion to endothelial cells via chemokine receptors and tyrosine phosphorylation signaling pathways.

Laboratory Investigation (2005) 0, 000–000. doi:10.1038/labinvest.3700256

Keywords: adhesion; chemokine receptors; endothelial cells; integrins; peripheral blood mononuclear cells; peptide hormones; tyrosine phosphorylation

Extravasation of leukocytes plays a central role in inflammatory and immune responses and is regulated by various signaling and adhesion molecules.^{1,2} Integrin-dependent adhesion of leukocytes to endothelial cells provides firm attachment and mediates transendothelial leukocyte migration. Chemokines produced in the local microenvironment stimulate the binding of integrins on leukocytes to their counter-ligands, which are expressed on endothelial cells and are upregulated by proinflammatory cytokines.^{1,2}

Prolactin (PRL), the hormone originally associated with milk production, is secreted by lymphocytes^{3–6}

and has stimulatory effects on the immune system.^{3–11} The presence of PRL receptors on immunocytes⁶ is consistent with PRL stimulation of T cells, B cells, natural killer cells, macrophages, neutrophils, CD34 + hematopoietic cells, and antigen-presenting dendritic cells.^{3–11} Animals with targeted disruption of either the PRL^{12,13} or the PRL receptor¹⁴ gene suggest that PRL is not essential for normal immune system development or function. However, PRL can be important for immune system homeostasis in autoimmune diseases and in stressful conditions including trauma, infection, and inflammation.^{15,16}

The role of female hormones in immune system homeostasis is supported by the well-established observations that many autoimmune diseases occur more frequently in women than in men,¹⁷ and that depression of immune functions following conditions of severe stress is lower in females than in males.¹⁸ In this regard, the circulating levels of PRL are higher in females and elevated in patients with

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Received 20 November 2004; revised 14 January 2005; accepted 18 January 2005; published online 00 month 00

systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, psoriatic arthritis, and in patients prior to transplant rejection.^{11,19–22} Moreover, PRL can protect against the suppression of immune functions under severe stress. For example, administration of PRL after trauma-induced hemorrhage improves macrophage and splenocyte functions,^{23,24} reduces plasma corticosterone levels, and decreases the mortality from subsequent sepsis.²⁴ In addition, the PRL receptor is upregulated in the thymus during acute phase response induced by LPS;²⁵ PRL protects against bacterial infections,^{26,27} and exposure to PRL can result in inflammatory responses characterized by infiltration of lymphocytes, macrophages, and neutrophils.^{28–30} The immunoenhancing properties of PRL may also include stimulation of leukocyte adhesion. PRL induces the expression of intracellular adhesion molecule (ICAM)-1 in the corpus luteum in association with enhancing the local accumulation of monocytes/macrophages.²⁹ Furthermore, PRL receptors belong to the hematopoietic cytokine receptor superfamily, characterized by its ability to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway.⁶ This pathway can be functionally linked to the focal adhesion kinase (FAK)/paxillin pathway involved in integrin-mediated cell adhesion and migration.^{31,32} In fact, PRL activates JAK-2/STAT-5 and induces tyrosine phosphorylation of FAK and of paxillin in breast carcinoma cells.³¹ Moreover, growth hormone, which is structurally related to PRL and has receptors belonging to the same superfamily,⁶ stimulates neutrophil adhesion through tyrosine phosphorylation of JAK-2, STAT-3, FAK, and paxillin.³²

This study was undertaken to investigate whether PRL stimulates integrin-mediated adhesion of leukocytes to endothelial cells. Herein, we demonstrate that PRL promotes the adhesion of peripheral blood mononuclear cells (PBMC) to human umbilical vein endothelial cells (HUVEC), and that this stimulation is mediated by the integrins leukocyte functional antigen-1 (LFA-1) (also known as CD11a/CD18) and very late antigen-4 (VLA-4) (or CD49d/CD29). Furthermore, we show that PRL up-regulates the expression of the chemokine receptor CXCR3 involved in the selective recruitment of T cells³³ and enhances tyrosine phosphorylation of paxillin in the Jurkat human T-leukemic cell line. These results reveal previously unrecognized proinflammatory actions of PRL.

Materials and methods

Antibodies and Reagents

Human recombinant PRL produced in C127 cells was from Genzyme Corporation (Framingham, MA, USA), whereas human recombinant PRL generated in *Escherichia coli* was from Joseph A Martial

(University of Liege, Sart Tilman, Belgium). Cell culture media and reagents and human plasma fibronectin (FN) was obtained from Gibco-BRL (Gathersburg, MD, USA). Polymixin-B and pertussis toxin were from SIGMA Chemicals (St. Louis, MO, USA). Na⁵¹CrO₄ was from Amersham Life Science (Buckinghamshire, UK). Soluble recombinant human ICAM-1, anti-LFA-1 (BCA1), and anti-VLA-4 (BBA37) mAb were from R&D Systems (Minneapolis, MN, USA). Anti-phosphotyrosine (4G10) and anti-paxillin (5H11) mAb were from Upstate Biotechnology (Lake Placid, NY, USA) and polyclonal Ab anti-Jak2, anti-STAT-3 and anti-STAT-5 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Genistein was from Calbiochem (San Diego, CA, USA).

Cell Isolation and Culture

PBMC were isolated from healthy, nonsmoker, male volunteers (aged 20–45 years) using a density gradient of Histopaque-1077 (Sigma Chemicals) according to the manufacturer's instructions. Cells were washed in PBS, resuspended at a final concentration of 10⁶ cells/ml in RPMI-1640 medium (Gibco-BRL) supplemented with 2 mM of L-glutamine, antibiotics and 10% FBS. PBMC were cultured for up to 3 days in the presence or absence of PRL (50–200 ng/ml) with or without polymixin B (10 µg/ml). For the 5 min to 1 h incubations, cells were resuspended in culture medium without serum. HUVEC were obtained from the umbilical vein and cultured on FN-coated dishes (1 µg/cm²) as described elsewhere.³⁴ Jurkat cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 1% penicillin–streptomycin, 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate. Jurkat cells were seeded at 2 × 10⁵ cells/ml and the medium was changed every 48 h.

Adhesion of PBMC to HUVEC

The adhesion method used was based on that described by Shimizu *et al.*³⁵ HUVEC (passages 3–6) were plated at 4 × 10⁴ cells per well in FN-coated 96-well plates and cultured for 48 h or until confluent. HUVEC were activated with 10 ng/ml of IL-1β for 4 h, washed and placed in assay medium (RPMI-1640 with 20 mM HEPES) immediately prior to the beginning of the experiment. PBMC were labeled for 1 h at 37°C with ⁵¹Cr (35 µCi per 5 × 10⁶ cells) before short-term (5–30 min) PRL treatment or after ending treatment with PRL for 1–18 h. The labeled PBMC were resuspended in assay medium, and added (5 × 10⁴ cells per well) to quintuplicate wells of endothelial cells in a final volume of 100 µl. PBMC and HUVEC were coincubated for 1 h at 37°C with or without blocking mAb anti-LFA-1 and anti-VLA-4 mAb at a saturating concentration of 10 µg/

ml. In other experiments, PBMC treated or not with PRL for 72 h were incubated in the presence or absence of pertussis toxin (1 mM) for 2 h, washed and labeled with ^{51}Cr before coincubation with IL-1 β -activated HUVEC. In addition, PBMC or Jurkat cells were labeled with ^{51}Cr and then incubated in the presence or absence of genistein (10 μM) for 30 min, followed by a 30-min incubation with or without PRL, before coincubation with activated HUVEC. After coincubation, wells were washed twice by immersion in 37°C Dulbeccó's PBS (D-PBS) containing 1% BSA, followed by flicking to remove nonadherent cells. HUVEC and bound PBMC were lysed with 1% Triton X-100 in assay medium, and γ -emissions were counted. Bound radioactivity was expressed as percentage of the total radioactivity of the PBMC added. The increase ratio is the ratio of the bound values in the treated sample to that in the untreated control.

Adhesion of PBMC to Immobilized FN or ICAM-1

Adhesion of PBMC to FN or ICAM-1 was evaluated as described previously.³⁶ Briefly, microtiter plates (Immulon II, Dynex Technologies, Chantilly, VA, USA) were coated for 1 h at room temperature with 50 μl of FN (100 $\mu\text{g}/\text{ml}$) or 50 μl of ICAM-1 (10 $\mu\text{g}/\text{ml}$) dissolved in D-PBS. The remaining protein-binding sites on the plastic surface were blocked by incubation for 1 h at room temperature with 10% BSA in D-PBS. Plates were washed 3 times by immersion in D-PBS. PBMC treated or untreated with PRL were added (5×10^4 cells/well) to quintuplicate FN- or ICAM-1-coated wells in a final volume of 100 μl . Cells were incubated for 30 min on ice and subsequently for 45 min at 37°C on a rotating platform. After incubation, wells were washed 3 times by immersion in D-PBS containing 10% BSA at 37°C to remove non-adherent cells. Bound cells were fixed with 4% paraformaldehyde in PBS for 10 min and stained with 0.1% toluidine blue. Adhesion was evaluated by counting bound cells in five microscope fields per well using the Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA). Results were normalized against untreated controls and are expressed as the increase ratio.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from PBMC using Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and treated with RNase-free DNase for 15 min at 37°C followed by phenol-chloroform extraction. Reverse transcription was performed using 5 μg of total RNA, 10 μM oligo(dT)₁₆ primers, and MMLV retrotranscriptase (Invitrogen Corporation) at 37°C for 60 min. For CXCR1 detection, forward and reverse primers were 5'-tcctgggaatgacacagca-3' and 5'-AAGCCAAAGGTGTGAGGCAG-3', respectively. For CXCR2, forward primer 5'-TTCCGAAGGACCGTC-

TACTCA-3' and reverse primer 5'-AGTTTGCTGTATTGTTGCCCATG-3' were used. For CXCR3, forward and reverse primers were 5'-AACTGTGGCCGAGAAAGCAG-3' and 5'-GCAGTGCATCTAGCCCAGG-3', respectively. mRNA for ribosomal protein L19 (RPL19) was amplified as internal control using as forward and reverse primers 5'-CGAAATCGCCAATGCCAACTC-3' and 5'-TGCTCCATGAGAATCCGCTTG-3', respectively. For I-TAC forward primer was 5'-ggttaccatcggagttta-caag-3' and reverse primer was 5'-aagtgtgatttgcata-gaaaaatgt-3'; for γ -interferon-inducible 10-kDa protein (IP-10) forward primer was 5'-aggggagcaaatcagatgcagt-3' and reverse primer was 5'-catc-cactagaacatagcacct-3'; and for MIG forward primer was 5'-catatgctctgaatttatctgagtc-3' and reverse primer 5'-aaaattattaaacctagtccacag-3'. Conventional PCR was performed as described³⁷ using 35 cycles for CXCR and CXCR3 ligand amplification and 21 cycles for the RPL19 internal control. Using RPL19 as internal standard, real-time PCR was carried out as indicated³⁷ in reactions that contained 1 μl aliquots of the reverse transcription reaction described above, 7.5 μl of Quantitect SYBR Green PCR (Qiagen, Hilden, Germany) and 400 nM forward and reverse primers in 15 μl total volume. Detection and data analysis were carried out on a LightCycler Instrument according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Immunoprecipitation and Western Blot

To investigate the tyrosine-phosphorylation of JAK-2, STAT-3, STAT-5, and paxillin, Jurkat cells (10×10^6) were stimulated with PRL (50–500 ng/ml) for 30 min at 37°C. Immunoprecipitation and Western blot analyses were performed as described elsewhere.³² Briefly, cells were lysed in 100 μl ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5% NP-40, 0.2% sodium deoxycholate, 100 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM sodium orthovanadate, and 1 mM NaF), and centrifuged (15 000 r.p.m.) at 4°C for 30 min to separate the insoluble fraction. Lysates were incubated overnight with 4 $\mu\text{g}/\text{ml}$ of anti-JAK-2, anti-STAT-3, anti-STAT-5, or anti-paxillin. Next, 20 μl of protein-A beads were added and the immunoprecipitated proteins were resolved on 12% SDS-PAGE. Membranes were probed with anti-phosphotyrosine mAb (0.5 $\mu\text{g}/\text{ml}$) and then reprobed with the respective mAb (1 $\mu\text{g}/\text{ml}$) to determine amounts of the proteins loaded on the gel.

Data Analysis

All results were replicated in three or more independent experiments. PBMC were from at least three different donors. Data are expressed as the mean \pm s.e.m. As appropriate, Student's unpaired *t*-

test or one-way ANOVA followed by Tukey's test to compare individual means was used for statistical comparisons. The significance level was set at 5%.

Results

PRL Increases PBMC Adhesion to HUVEC

Freshly isolated PBMC were treated with PRL for up to 72 h and incubated for 1 h with HUVEC activated

by pretreatment with IL-1 β for 4 h. PRL stimulated the adhesion of PBMC to HUVEC as early as 5 min after treatment (Table 1). Maximal adherence was observed at 1 h and was maintained thereafter. PBMC adhesion showed a bell-shaped response to treatment with increasing concentrations of recombinant PRL for 1 h (Figure 1a) or 72 h (Figure 1b). Peak stimulation was observed at 50 ng/ml and produced a 60 and 67% increase of adhesion to HUVEC after 1 and 72 h of PRL treatment, respec-

Table 1 Effect of PRL on PBMC adhesion to HUVEC^a

	Time						
	5 min	15 min	30 min	1 h	6 h	18 h	72 h
Control	20.6 \pm 0.67	21.5 \pm 0.95	19.7 \pm 1.2	17.2 \pm 0.81	14.3 \pm 0.62	12.5 \pm 0.47	9.2 \pm 0.76
PRL (50 ng/ml)	27.5 \pm 0.82*	30.3 \pm 0.86*	27.3 \pm 0.62*	28.2 \pm 1.33*	22.5 \pm 1.5*	19.4 \pm 1.71*	15.4 \pm 1.24*
Increase ratio	1.33	1.41	1.39	1.64	1.57	1.55	1.67

^aValues are bound radioactivity expressed as percentage of the total radioactivity in plated PBMC. Increase in ratio represents the change in the normalized values. PBMC were treated with PRL (50 ng/ml) according to the indicated time interval at 37°C. PBMC were then labeled with ⁵¹Cr and incubated with IL-1 β -activated HUVEC for 1 h. Nonadherent cells were washed and total radioactivity per well counted. Results are presented as the mean \pm s.e.m. of three separate experiments.

* P <0.05 vs untreated cells.

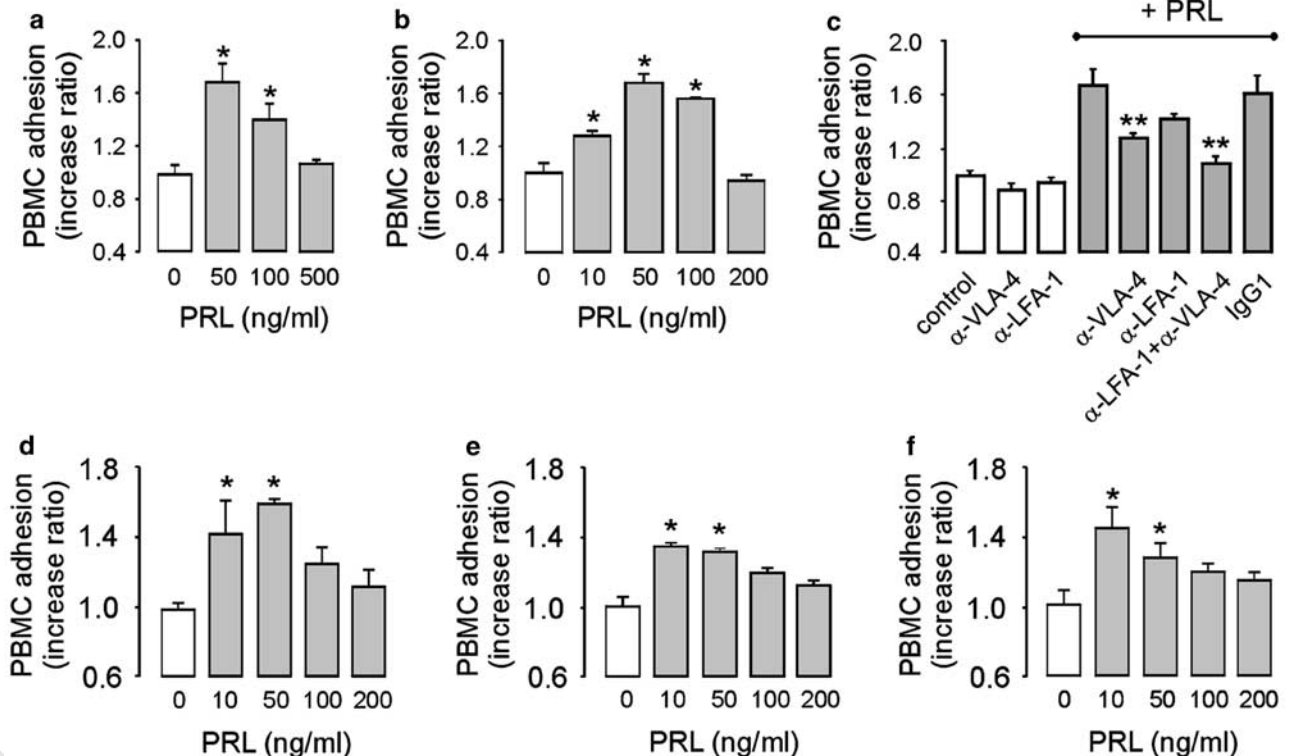


Figure 1 PRL stimulates the adhesion of PBMC to HUVEC through integrins VLA-4 and LFA-1. Dose-dependent effect of a 1 h (a) or 72 h (b) treatment with PRL on the adhesion of PBMC to HUVEC. PBMC were incubated with medium alone or with the indicated concentration of PRL and coincubated with IL-1 β -activated HUVEC. (c) Inhibitory effect of anti-LFA-1 and anti-VLA-4 mAb on PRL-induced PBMC adhesion to HUVEC. PBMC were treated for 72 h with or without PRL (50 ng/ml) in the absence or presence of 10 μ g/ml of each mAb alone or in combination or with control Ab (IgG1). (d, e) Dose-dependent effect of incubating with PRL for 72 h on the adhesion of PBMC to immobilized ICAM-1 (d) or FN (e). (f) PRL-induced adhesion to FN was evaluated in the presence or absence of polymyxin-B (10 μ g/ml). The increase ratio is the ratio of the bound values in the treated sample to that in the untreated control. Results are the means \pm s.e.m. of three independent experiments. * P <0.05 vs nontreated control. ** P <0.05 vs PBMC treated with PRL in the absence of mAb.

tively. Similar dose–response curves were obtained with recombinant PRL generated in C127 cells (Figure 1) or in *E. coli* (data not shown). In all the following experiments, C127 recombinant PRL was used.

LFA-1 and VLA-4 Integrins Mediate PRL Stimulation of PBMC Adhesion to HUVEC

To determine whether integrins VLA-4 and LFA-1 mediate PRL stimulation of adhesion, the effect of function-blocking mAb to VLA-4 and LFA-1 was tested upon the 72-h PRL stimulation of PBMC adhesion to HUVEC (Figure 1c). Anti-VLA-4 mAb reduced PRL-induced adhesion by 58% ($P < 0.05$), while anti-LFA-1 mAb produced a smaller nonsignificant inhibition. However, the combined use of both anti-VLA-4 and anti-LFA-1 mAb inhibited PRL stimulation by 83%. In addition, PRL stimulated the adhesion of PBMC to immobilized ICAM-1 or FN, ligands of VLA-4 and LFA-1, respectively. In these experiments, PBMC treated with PRL for 72 h were incubated for 1 h with the purified, immobilized ligands. PRL stimulated PBMC adhesion in a bell-shaped dose–response fashion, with maximal increases of 60 and 40% for ICAM-1 (Figure 1d) and FN (Figure 1e), respectively.

Because bacterial LPS are a widespread contaminant, and are known to stimulate leukocyte adhesion to endothelial cells and extracellular matrix proteins,² we ascertained the effect of PRL in the presence of polymyxin B, an inhibitor of LPS activity. Polymyxin B did not interfere with PRL-induced stimulation of PBMC adhesion to FN (Figure 1f). This finding is consistent with activity displayed by PRL from different sources (C127 cells and *E. coli*) and suggests that PRL alone is an effective stimulator of leukocyte adhesion.

PRL Induces the Expression of the Chemokine Receptor CXCR3 in PBMC

To study the putative contribution of chemokine receptor pathways to PRL-induced stimulation of PBMC adhesion, we investigated the effects of PRL on the expression of chemokine receptors CXCR3, CXCR2 and CXCR1, which selectively bind chemokines sharing the CXC arrangement of their first two cysteine residues. Total RNA from PBMC incubated with or without PRL for 72 h was subjected to reverse transcription-polymerase chain reaction (RT-PCR). Amplification yielded products with the expected lengths for CXCR3 (68 bp), CXCR2 (73 bp), and CXCR1 (64 bp). The expression of the CXCR3 transcript was barely detected without PRL and markedly induced by PRL treatment (Figure 2a). PRL induced CXCR3 mRNA by 2.5-fold when evaluated by real-time PCR (Figure 2b). In contrast, the expression of CXCR2 and CXCR1 transcripts was similar in the presence or absence of PRL (Figure

2a). In addition, when carried out in HUVEC using primers for CXC chemokines, RT-PCR amplified products with the expected lengths for IP-10 (263 bp), I-TAC (295 bp), and MIG (335 bp) (Figure 2c), suggesting that HUVEC express the three known ligands of CXCR3. Finally, pertussis toxin, a G-protein inhibitor, blocked PRL stimulation of PBMC adhesion to HUVEC (Figure 2d), further suggesting

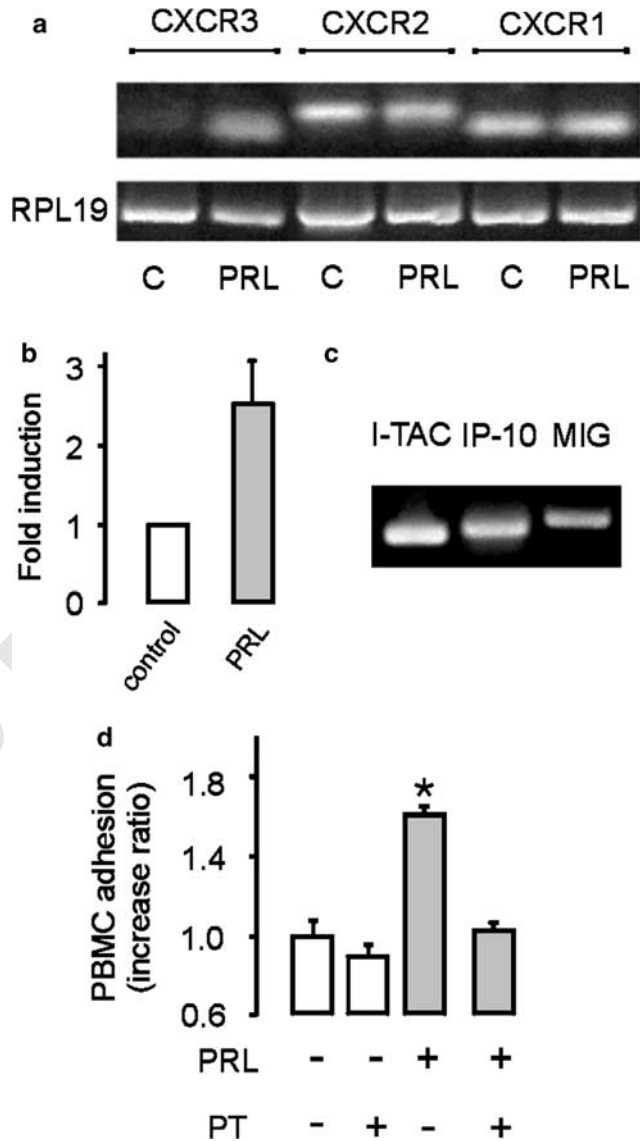


Figure 2 PRL induces the expression of chemokine receptor CXCR3 in PBMC. (a) mRNAs for chemokine receptors: CXCR3, CXCR2, and CXCR1 were analyzed by RT-PCR performed on total RNA extracted from PBMC incubated for 72 h in the presence or absence of PRL (50 ng/ml). Amplification of RPL-19 is shown as control. (b) Quantitation of CXCR3 mRNA expression using real-time PCR. (c) mRNAs for the CXCR3 ligands I-TAC, IP-10, and MIG were analyzed by RT-PCR in HUVEC. (d) Inhibitory effect of pertussis toxin on PRL-induced adhesion of PBMC to HUVEC. PBMC treated or not with PRL for 72 h were incubated in the presence or absence of pertussis toxin (1 mM) for 2 h before cocultivation with HUVEC. Results are means \pm s.e.m. * $P \leq 0.05$ vs nontreated control.

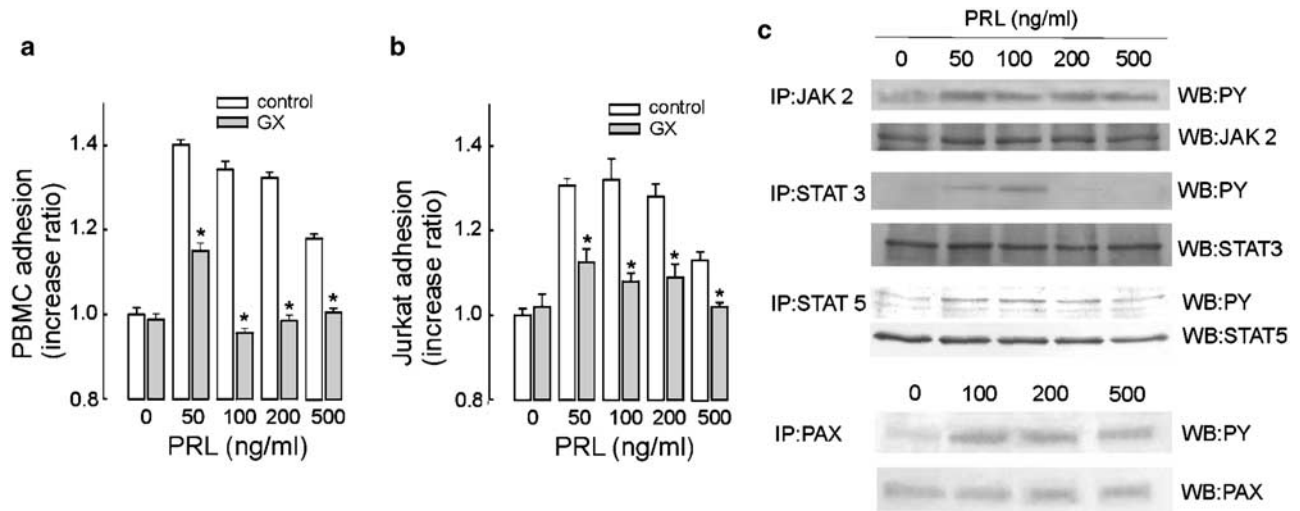


Figure 3 PRL stimulates adhesion through tyrosine phosphorylation pathways. Inhibitory effect of genistein on the PRL-induced adhesion of PBMC (a) or Jurkat cells (b) to HUVEC. Cells were incubated in the presence or absence of genistein (10 μ M) for 30 min, followed by a 30-min incubation with or without increasing concentrations of PRL before incubation with HUVEC. Results are means \pm s.e.m. * $P \leq 0.05$ vs nontreated control at the same PRL concentration. (c) PRL induces tyrosine phosphorylation of JAK-2, STAT-3, STAT-5, and paxillin (PAX) in Jurkat cells. Jurkat cells were treated with the indicated concentrations of PRL for 30 min and cell lysates immunoprecipitated (IP) with Ab anti-JAK-2, STAT-3, STAT-5, or PAX. Subsequently Western blots (WB) were probed with antiphosphotyrosine mAb or with Ab anti-JAK-2, STAT-3, STAT-5, or PAX as described in Materials and methods.

the contribution of the G-protein coupled receptor CXCR3 to PRL-induced adhesion.

PRL Stimulates Adhesion Through Tyrosine Phosphorylation Pathways

We also tested whether tyrosine-phosphorylation pathways may contribute to the PRL effect on adhesion by using genistein, a typical tyrosine kinase inhibitor. In these experiments, PBMC or the Jurkat T-cell line were incubated with genistein for 30 min and then with or without PRL for 30 min. Subsequently, all cells were incubated with activated HUVEC for 1 h. Consistent with previous findings, treatment with PRL for 30 min stimulated the adhesion of PBMC and Jurkat cells to HUVEC in a bell-shaped dose-dependent fashion. Genistein blocked the effect of PRL in both PBMC and Jurkat cells (Figure 3a,b). Next, the effect of PRL on tyrosine phosphorylation of JAK-2, STAT-3, STAT-5, and paxillin was investigated. Jurkat cells were incubated with different concentrations of PRL for 30 min and subjected to immunoprecipitation-Western blot. Initial blotting with antiphosphotyrosine antibodies showed that PRL induced the tyrosine phosphorylation of JAK-2, STAT-3, STAT-5, and paxillin (Figure 3c). Subsequent blotting with the respective antibodies demonstrated that equal amounts of these proteins were loaded in each lane.

Discussion

Extensive evidence supports the stimulatory role of PRL in the immune-hematopoietic system,³⁻¹¹ and although an absolute requirement for PRL in

immune function has been questioned,¹²⁻¹⁴ new interest has been raised by evidence showing that PRL can counteract the effects of negative immunoregulatory factors in autoimmune diseases and in stressful conditions.^{15,16,38,39} Here, we report that PRL promotes the adhesion of leukocytes to vascular endothelium, a previously unrecognized action that may contribute to the immunoenhancing properties of this hormone during immune-inflammatory processes.

The results of this study indicate that PRL rapidly augments the adhesion of PBMC to IL-1 β -activated HUVEC and that this effect is sustained for many hours. Maximal stimulation (67%) is lower than the reported increase (220%) in the adhesion of splenocytes to HUVEC induced by IL-2,⁴⁰ but higher than the increase (32%) in neutrophil adhesion induced by growth hormone,³² a PRL-related hormone with well-known immune functions.⁴¹ Similar to the effect of growth hormone on cell adhesion,³² the dose-response curve of PRL on leukocyte adhesion was 'bell-shaped.' Such a shape is a common observation for PRL stimulation of the PRL receptor,⁴ and it has been explained on the basis that signal transduction by PRL requires binding of one PRL molecule to two receptor molecules.⁴² At high concentrations, PRL can saturate the receptor and hinder receptor dimerization.

While this is the first report of PRL acting directly on leukocytes to stimulate their adhesion to vascular endothelium, the ability of PRL to recruit immune cells has been reported previously. *In vivo* studies have shown that PRL stimulates the accumulation of mononuclear cells in the prostate during inflammatory reactions,²⁸ in the pleural cavity after carrageenan injection,³⁰ and in the corpus luteum during

regression.²⁹ Also, PRL stimulates the expression of ICAM-1 in the corpus luteum,²⁹ and *in vitro* studies showed that PRL promotes the chemotactic capacity of macrophages.⁴³

Stimulation of cell adhesion by PRL may involve the activation of integrins LFA-1 and VLA-4, major molecular pathways that mediate firm attachment of leukocytes to vascular endothelium.^{1,2} Circulating leukocytes constitutively express LFA-1 and VLA-4 in a low-affinity state. However, after leukocyte activation, the affinity of both integrins is enhanced, enabling binding to their counter-ligands on endothelial cells, that is, ICAM-1 and ICAM-2 for LFA-1, and vascular cell adhesion molecule-1 (VCAM-1) for VLA-4.^{1,2} Consistent with LFA-1 and VLA-4 being crucial determinants in PRL-stimulated adhesion, the combination of mAbs against both LFA-1 and VLA-4 blocked PRL-induced adhesion of PBMC to HUVEC, and PRL stimulated the adhesion of PBMC to immobilized ICAM-1 and FN.

To analyze how PRL becomes functionally linked to integrin-mediated adhesion, we investigated the possible association of PRL with chemokine receptor pathways. Chemokines constitute a large family of chemotactic cytokines that are present at or near the endothelial cell surface and act by binding to G protein-coupled receptors on leukocytes to promote their integrin-mediated adhesion to endothelial cells. The CXC chemokine subfamily selectively activates receptors CXCR1 to CXCR6.⁴⁴ As reported for various leukocyte subtypes,⁴⁵ PBMC constitutively expressed the mRNA for CXCR1, CXCR2, and CXCR3; however, treatment with PRL resulted in the induction of CXCR3 but not of CXCR1 or CXCR2. CXCR3 is upregulated by IL-2 in T lymphocytes,⁴⁶ IL-2 promotes T-cell adhesion to vascular endothelium,⁴⁰ and it has been claimed that CXCR3 plays a major role in T-cell recruitment.³³ Also, the CXCR3 ligands IP-10, I-TAC, and MIG are upregulated in endothelial cells by $\text{INF-}\gamma$ ⁴⁷ and CXCR3 appears to be their only receptor on T cells.^{33,48} Therefore, T cells are likely targets for PRL-induced CXCR3 expression.

Upregulation of CXCR3 in T cells is a potential mechanism by which PRL could promote the adhesion of PBMC to vascular endothelium. Nearly, 70% of PBMC cells are T cells, and activation of CXCR3 promotes the transendothelial migration of T cells via the LFA-1 and VLA-4 pathways.⁴⁹ Also, HUVEC express the mRNAs for IP-10, I-TAC, and MIG,⁴⁷ and therefore can produce CXCR3 ligands in culture. More importantly, we found that the effect of PRL on PBMC adhesion to HUVEC was blocked by treatment with pertussis toxin, an inhibitor G-protein coupled receptors. Because all known chemokine receptors are coupled to G-proteins,³³ this result does not imply that CXCR3 is the only chemokine receptor mediating PRL stimulation of cell adhesion, only that chemokine receptors are likely to be involved.

G-protein coupled receptors elicit the rapid activation of integrins by complex mechanisms that

involve regulation of the affinity of integrins via conformational changes of integrin chains and regulation of the avidity of integrins by increasing lateral mobility and clustering. Signaling occurs through different types of second messengers including small GTPases of the RAS family (RAP1, RAPL, RHOA), cytoskeletal proteins (talin, paxillin), protein serine kinases (atypical protein kinase- α , phosphoinositide-3-kinase), and protein tyrosine kinases,⁵⁰ etc.⁵¹

While *de novo* protein synthesis of chemokine receptors may participate in the stimulation of leukocyte adhesion by PRL, a shorter-term mechanism may operate as well since PRL stimulates the adhesion of PBMC to HUVEC as early as 5 min after treatment. The PRL receptor signals through the JAK/STAT pathway, and activation of this pathway by PRL can be functionally linked to the increased tyrosine phosphorylation of paxillin.³¹ Paxillin is a signaling adaptor protein that binds to the cytoplasmic tails of integrins and is important for coordinating the formation of focal adhesions leading to integrin-mediated cell adhesion and motility.^{52,53} For example, tyrosine phosphorylation of paxillin stimulates LFA-1-dependent migration by promoting the activation of Pyk2 and FAK.⁵⁰

The present study demonstrates that treatment with PRL for 30 min triggers the tyrosine phosphorylation of JAK-2, STAT-3, STAT-5, and paxillin in the Jurkat-T human leukemic cell line, a PRL target cell.⁵⁴ This finding is consistent with extensive evidence showing that PRL stimulates tyrosine phosphorylation of JAK-2, STAT-3, and STAT5 in other cell types.^{5,6,55} Although PRL-induced tyrosine phosphorylation of JAK-2, STAT-5, paxillin, and FAK has been reported in breast cancer cells,³¹ this is the first study showing the activation of the JAK/STAT/paxillin pathway in immune cells. The molecular mechanism(s) linking the JAK/STAT pathway to the activation of the FAK/paxillin pathway remain largely unknown, but it has been proposed that STATs can function as intracellular adaptors in coupling the two pathways.³² STAT-3 is physically associated with JAK-2 and FAK in neutrophils, and the fact that growth hormone stimulates this association appears to contribute to the activation of the JAK-2/STAT-3/FAK/paxillin pathway by growth hormone.³² Finally, the possibility that PRL-induced leukocyte adhesion to endothelial cells is related to the tyrosine phosphorylation of these signaling molecules is suggested by the fact that genistein inhibits PRL-stimulated adhesion of Jurkat cells and PBMC to HUVEC. This observation is consistent with the previous report showing that growth hormone-mediated tyrosine phosphorylation of the JAK-2/STAT-3/FAK/paxillin pathway is inhibited by genistein, which also blocks the adhesion of neutrophils.³²

The fact that PRL stimulates adhesion of leukocytes to vascular endothelium is consistent with its

proposed role of enhancing inflammatory responses.^{11,15} Fluctuations in the circulating levels of PRL could stimulate immune function by this and other mechanisms. A wide range of stressors stimulate the release of PRL by the anterior pituitary gland,^{4,38,56} and some results indicate that PRL can counteract the negative effects of steroids or other suppressive agents also released by stress.^{16,38,39} In addition, it has been suggested that hyperprolactinemia during pregnancy and lactation can contribute to the adaptations required for immunological acceptance of the fetus and protection of the maternal immune system against general suppression.¹⁶ Finally, increased levels of PRL are associated with aggravated autoimmune diseases, whereas inhibition of PRL release by bromocriptine ameliorates autoimmunity progression.^{11,19–21} Owing to its sexually dimorphic characteristics, PRL actions on the immune system may contribute to the distinct immune environment that determines the female preponderance of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus.¹⁷

On the other hand, leukocyte adhesion may be stimulated not only by circulating PRL but also by the locally produced hormone. PRL is synthesized by various cells of the immune system,⁴ including PBMC⁵⁷ and by endothelial cells from different blood vessels,^{58,59} including HUVEC.³⁴ The putative contribution of locally produced PRL implies that the immunoenhancing properties of the hormone may not depend on changes in its circulating levels, but on specific regulatory mechanisms adjust PRL expression locally.

In summary, this work reports for the first time that PRL acts on circulating immune cells to stimulate their integrin-mediated adhesion to vascular endothelium. Elucidation of the precise cellular and molecular mechanisms by which PRL promotes leukocyte adhesion and the signaling pathways involved should be relevant for understanding the role of this hormone in inflammatory responses. This knowledge may help clarify gender differences in autoimmunity and could lead to the discovery of improved therapies for autoimmune diseases.

Acknowledgement

We thank A Prado, D Mondragón, P Galarza, M Garcia, and A Lara for their expert technical assistance and DD Pless for editing the manuscript. This work was supported by National Autonomous University of Mexico Grants IN227502 and the National Council of Science and Technology of Mexico Grant 36041-N and 43401.

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APENDICE 2

Expression of Prolactin Gene and Secretion of Prolactin by Rat Retinal Capillary Endothelial Cells

Alejandra Ochoa, Pável Montes de Oca, Jose Carlos Rivera, Zulma Dueñas, Gabriel Nava, Gonzalo Martínez de la Escalera, and Carmen Clapp

PURPOSE. Prolactin fragments inhibit blood vessel formation, whereas anti-prolactin antibodies induce angiogenesis in the cornea. Endothelial cells from brain capillaries and the umbilical vein produce prolactin, and this study was undertaken to determine whether retinal capillary endothelial cells could be a source for prolactin in the eye.

METHODS. Primary cultures of rat retinal endothelial cells were investigated for the expression of prolactin mRNA by reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis and by *in situ* hybridization. The prolactin protein was analyzed by immunocytochemistry, enzyme-linked immunosorbent assay, Western blot analysis, and the Nb2-cell bioassay. The effect of prolactin and the 16-kDa prolactin fragment on retinal endothelial cell proliferation was investigated, and the expression of the cloned prolactin receptor was analyzed by RT-PCR and Southern blot analysis.

RESULTS. Retinal endothelial cells expressed prolactin mRNA and full-length 23-kDa prolactin. Prolactin was observed in the cytoplasm of cells and in their conditioned medium at levels 300 times those described in endothelial cells from other vessels and species. Exogenous 16-kDa prolactin inhibited rat retinal endothelial cell proliferation, whereas 23-kDa prolactin was inactive. No evidence was obtained for the expression of the cloned prolactin receptor in these cells, but the prolactin receptor was amplified in whole rat retina.

CONCLUSIONS. Endothelial cells from the microcirculation of rat retina produce and release prolactin. That the cloned prolactin receptor was not expressed in these cells argues against direct autocrine effects of prolactin. Possible paracrine effects are suggested by the expression of the prolactin receptor in retinal tissue. (*Invest Ophthalmol Vis Sci.* 2001;42:1639-1645)

Angiogenesis, the formation of new capillary blood vessels, is a leading cause of blindness worldwide and occurs in diseases such as diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, corneal conjunctivalization, and ocular trachoma.¹ Ocular angiogenesis may result from an imbalance between stimulatory and inhibitory factors presumed to occur from an elevated expression of local angiogenic factors induced by hypoxia.^{2,3}

Various angiogenic factors have been proposed to mediate vasoproliferative eye diseases, including basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1 and, most importantly, vascular endothelial growth factor (VEGF).⁴⁻⁷ However, the imbalance responsible for pathologic angiogenesis may also result from downregulation of inhibitors of neovascularization.⁸⁻¹⁰

Several of the described inhibitors of angiogenesis are fragments of larger proteins, including angiostatin, a 36-kDa internal fragment of plasminogen¹¹; endostatin, the 20-kDa C-terminal fragment of collagen XVIII¹²; an internal fragment of platelet factor 4¹³; fragments of laminin¹⁴; peptides derived from thrombospondin¹⁵; and the 16-kDa and 14-kDa N-terminal fragments of prolactin (PRL).^{16,17}

PRL is a pleiotropic protein that acts on functions that range from reproduction and osmoregulation to immunomodulation and angiogenesis.¹⁸ PRL fragments of 16 and 14 kDa appear to be produced by a cathepsin-D-like protease¹⁹ and have been reported in the anterior and posterior lobes of the pituitary gland and in the circulation.^{17,20} These PRL fragments act as potent antiangiogenic factors *in vivo* and *in vitro*, inhibiting endothelial cell proliferation,^{16,21} and stimulating type 1 plasminogen activator inhibitor expression.²² These inhibitory actions appear to occur through a receptor distinct from cloned PRL receptors, because specific, high-affinity, saturable binding sites for 16-kDa PRL are found in endothelial cells.²³

PRL fragments may be involved in the control of ocular angiogenesis. The 16-kDa PRL inhibits bFGF-induced corneal neovascularization, and implants containing anti-PRL antibodies induce a local angiogenic reaction in the cornea.²⁴ Similarly, PRL has been measured in the cornea and aqueous humor of the rat²⁵ and in the aqueous humor and subretinal fluid of patients with retinopathy of prematurity.²⁶ Some of this PRL may be produced locally within the eye. Reverse transcription-polymerase chain reaction (RT-PCR) has detected the expression of PRL mRNA in the retina of the rat²⁵ and in the vitreous fibrovascular membranes of patients with retinopathy of prematurity.²⁶ Endothelial cells from bovine brain capillaries and the human umbilical vein produce PRL,^{27,28} and we investigated whether endothelial cells from the microcirculation of the retina secrete PRL and thus could constitute a source for ocular PRL. A preliminary report of these findings has been presented.²⁹

MATERIALS AND METHODS

Reagents

1,1'-Dioctadecyl-1,3,3,3'-tetramethylindocarbocyanine perchlorate acetylated low-density lipoprotein (DiI-Ac-LDL) was purchased from Molecular Probes, Inc. (Eugene, OR); fluorescein isothiocyanate (FITC)-conjugated *Bandeiraea simplicifolia* I isolectin B₄ (BSI) and tetramethylrhodamine isothiocyanate (TRITC)-labeled *Ulex europaeus* I (UEA I) lectin from Sigma Chemical Co. (St. Louis, MO); and monoclonal antibody against human von Willebrand protein from Accurate Chemical & Scientific Corp. (Westbury, NY). VEGF was a kind gift from Napoleone Ferrara (Genentech, San Francisco, CA), and human bFGF

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Supported by Grant 55000595 from The Howard Hughes Medical Institute, Grants 27950-N and 34309M from the National Council of Science and Technology of Mexico, and Grant IN226799 from the National University of Mexico.

Submitted for publication August 24, 2000; revised January 12, 2001; accepted January 31, 2001.

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was kindly provided by Judith A. Abraham (Scios, Inc., Mountain View, CA). Normal rabbit serum and second antibodies against mouse or rabbit IgG coupled to TRITC were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rat 23-kDa PRL (biological grade) and rat PRL antisera (S-9 and IC-5) were donated by Albert F. Parlow of the National Hormone and Pituitary Program (NHPP, Torrance, CA). Locally produced anti-PRL antiserum was raised in rabbits against rat 23-kDa PRL standard and characterized as described.²⁴ The 16-kDa PRL was generated after the enzymatic proteolysis of rat 23-kDa PRL with a particulate fraction from rat mammary glands, gel filtration, and carbamidomethylation, as reported.³⁰

Isolation and Culture of Rat Retinal Capillary Endothelial Cells

Rat retinal capillary endothelial cells (RRCECs) were obtained from rat retinas using a modified method described in rabbits.³¹ All animals were maintained and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyeballs were hemisected, and the vitreous placed on fibronectin-coated plates (10 μ g/ml; obtained as the rest of the reagents for tissue culture from Gibco BRL, Rockville, MD). Retinas cut into small pieces were placed in the vitreous-containing plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), porcine heparin (100 μ g/ml), bFGF (2 ng/ml), and penicillin-streptomycin (100 U/ml). Because endothelial cells are quick to adhere, their selection versus other cell types was favored by frequent changes of culture medium—initially, 3 hours after the onset of culture and then every 12 hours. Retinal explants were removed on the third day of culture. On days 7 through 10, round cells forming patches with a cobblestone appearance were picked up using a micropipette containing 0.025% trypsin and transferred to a 48-well plate coated with fibronectin. The cells were grown and subsequently split 1:3. Typically, three to six rats (250 g each) provided enough material for one 60-mm plate. Cells were stored frozen by passage 4 or replated for experimental use between the 5th and the 12th passages.

RRCEC Proliferation

RRCECs (2.5×10^3 cells/15-mm well) were cultured in serum-free Opti-MEM (Gibco BRL), except for VEGF proliferation experiments in which the Human Endothelial-Serum Free Medium System (Gibco BRL) supplemented with epidermal growth factor (EGF, 10 ng/ml) was used. Incubations were for 48 hours, with the growth factors or PRLs added twice: once at the time of seeding the cells and once again 24 hours later. At the end of the incubation, cells were pulsed for 20 hours with 0.6 μ Ci [³H]-thymidine/15-mm well, and [³H]-thymidine incorporation into DNA was measured as an index of cell proliferation.²¹

Immunocytochemistry, Lectin-Binding and Dil-Ac-LDL Uptake

RRCEC grown on glass coverslips previously coated with fibronectin in Opti-MEM were washed with PBS and fixed in 4% formaldehyde-PBS for 10 minutes, at room temperature (RT). Immunocytochemistry was performed as described²⁸ using a monoclonal antibody against the von Willebrand factor (1 μ g/ml), or anti-PRL antiserum (1:1000; IC-5) and a 1:100 dilution of second antibodies coupled to rhodamine. For lectin-binding experiments, cells were incubated for 30 minutes with either BSI (25 μ g/ml) or UEA-I (100 μ g/ml) in PBS supplemented with 0.1 mg/ml CaCl₂ and MgCl₂, as described.^{32,33} For Dil-Ac-LDL uptake, live cells on coverslips were incubated for 4 hours with Dil Ac-LDL (10 μ g/ml) in 10% FBS-DMEM, as indicated,³⁴ and fixed as for immunocytochemistry. In all cases, cells were coverslipped using an anti-fade kit (Molecular Probes, Inc.) and examined under an epifluorescence microscope (model BX60; Olympus, Lake Success, NY).

Endothelial Cell Networks

Formation of cell networks was investigated by plating RRCECs within type I collagen gels (Vitrogen 100; Collagen Corp., Palo Alto, CA), as reported.¹⁶

Reverse Transcription–Polymerase Chain Reaction

RT-PCR and Southern blot analysis were performed essentially as described.³⁵ For rat PRL detection, four primers complementary to exons 2 to 5 of the rat PRL gene, 30 cycles, and an annealing temperature of 65°C were used, as previously indicated.¹⁷ For rat PRL receptors, primers and conditions were as previously reported.³⁶ Briefly, a common forward primer (5'-ATCCTGGGACAGATGGAGGAC-3') and a common reverse primer (5'-ATCCACACGGTTGTGTCCTTC-3') were used to detect the short, intermediate, and long isoforms. Reverse primers were used to specifically detect the short (5'-TGGCTGAGGCTGACAAAAGAG-3') or long (5'-AGACAGTGGGGCTTTTCTCCT-3') isoforms. Amplification was with 40 cycles and an annealing temperature of 56°C.

In Situ Hybridization

Sense and antisense probes were transcribed *in vitro* from linearized plasmids (pcDNA3; Invitrogen, Carlsbad, CA) containing the cDNA for rat PRL with T7 and SP6 polymerases and labeled with digoxigenin-uridine triphosphate (Boehringer-Mannheim, Mannheim, Germany). RRCECs grown on fibronectin-coated coverslips were fixed with 4% formaldehyde, 5% acetic acid, and 0.9% NaCl-PBS at room temperature for 30 minutes. *In situ* hybridization was performed according to the manufacturer's instructions. Briefly, cells were dehydrated with ethanol, washed in 100% xylene to remove residual lipids, and rehydrated. Cells were treated with 0.1% pepsin in 0.1 N HCl, postfixed with 1% formaldehyde, washed, and prehybridized for 1 hour at 37°C in hybridization buffer (4 \times SSC [1 \times 150 mM NaCl/15 mM sodium citrate, pH 7.0], 10% dextran sulfate, 1 \times Denhardt's solution, 2 mM EDTA, 50% formamide, and 500 μ g/ml herring sperm DNA). Probes were denatured at 80°C for 10 minutes and hybridization performed in hybridization buffer for 16 hours at 37°C. Cells were washed with 60% formamide in 0.2 \times SSC at 37°C and with 2 \times SSC at room temperature. Hybridized probe was determined by using the fluorescent antibody enhancer set for digoxigenin detection (Boehringer-Mannheim) and viewed with a microscope with an attached confocal system (PCM 2000; Nikon, Melville, NY).

RRCEC Lysates and Conditioned Media

RRCECs (10^6 cells/100-mm well) were incubated for 24 hours in 10 ml serum-free Opti-MEM. Conditioned media were clarified by centrifugation (10 minutes at 1200g), concentrated 10 times (Centricon 3; Amicon, Beverly, MA) and stored at -70°C. Cells were lysed in 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 μ g/ml aprotinin, and 100 μ g/ml phenylmethylsulfonyl fluoride (Sigma, Milwaukee, WI).

Western Blot Analysis

Two micrograms of protein from RRCEC lysates and conditioned media were mixed and boiled in electrophoresis sample buffer containing β -mercaptoethanol, and resolved in an SDS-polyacrylamide slab gel (15% acrylamide-bisacrylamide). Gels were blotted onto nitrocellulose membranes, probed with a 1:500 dilution of an anti-PRL antiserum (NHPP, S-9 or locally produced), and developed using the alkaline phosphatase second antibody kit (Bio-Rad Laboratories, Hercules, CA).

Enzyme-Linked Immunosorbent Assay

The ELISA was performed as described elsewhere,²⁴ using wells coated with 10 ng of 23-kDa PRL, a 1:8000 dilution of locally raised anti-PRL antiserum, and a 1:2000 dilution of horseradish peroxidase (HP)-conjugated second antibodies (Vector Laboratories, Burlingame, CA). Bound HP-conjugated antibodies were revealed by reaction with o-phenylenediamine dihydrochloride in the presence of hydrogen peroxide. Optical density was measured at 490 nm.

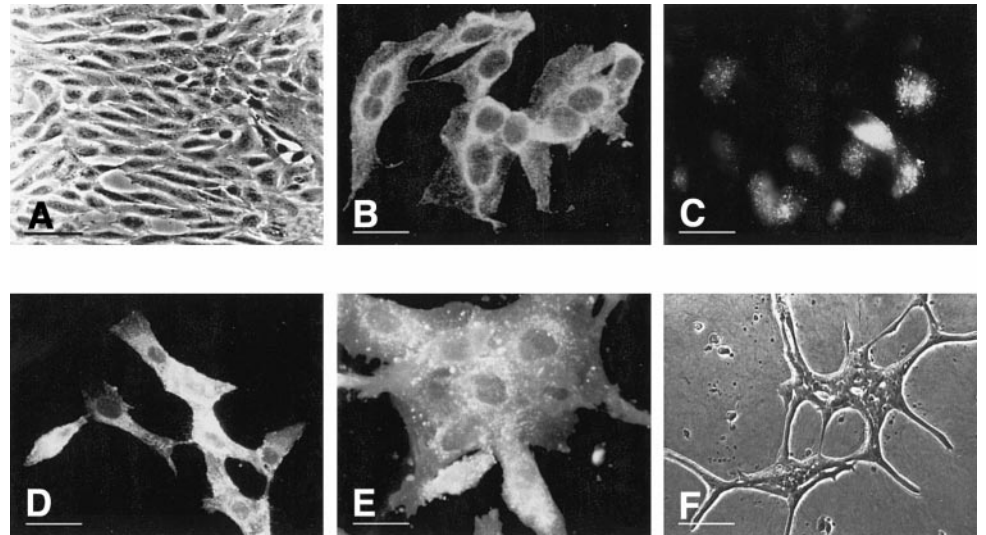


FIGURE 1. Characterization of RRCEC cultures. RRCECs obtained after sequential seeding and cloning fulfilled the following endothelial cell criteria: they formed monolayers (A), showed positive immunostaining for the von Willebrand protein (B), incorporated fluorescent Dil-Ac-LDL (C), reacted with fluorescent endothelial cell-specific BS-I (D) and UEA-I (E), and formed networks when grown on type I collagen gels (F). Scale bar, (A, F) 38 μm ; (B-E) 21 μm .

Nb2-Cell Bioassay

Bioactive PRL was determined in RRCEC-conditioned media by using the Nb2-cell bioassay, as detailed previously.³⁷ Incubations were performed for 48 hours in the absence or presence of different dilutions of RRCEC-conditioned medium, nonconditioned medium, or 23-kDa PRL standard, with or without a 1:500 dilution of locally produced PRL antiserum. Nb2-cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.³⁸

Statistical Analysis

Each experiment was an average of three or more replicates of each condition. Results are representative of three or more experiments. The data in each experiment were analyzed for statistical significance by Student's *t*-test.

RESULTS

Characterization of RRCEC Cultures

RRCECs fulfilled various established criteria³⁹ for the identification of endothelial cells—that is, they formed monolayers with cobblestone morphology and showed positive immunofluorescent staining for von Willebrand protein and a strong uptake of Dil-Ac-LDL (Figs. 1A, 1B). Similarly, the RRCECs bound the endothelial cell-specific lectins BS-I and UEA-I and associated into cell networks when grown within type I collagen gels (Figs. 1C–F). Furthermore, RRCECs proliferated in response to increasing concentrations of the angiogenic factor bFGF and the specific endothelial cell mitogen VEGF (Fig. 2).

Expression of PRL mRNA by RRCECs

Total RNA from RRCECs was subjected to RT-PCR in which four combinations of primers were used with annealing sites within exons 2 to 5 of the rat PRL gene (Fig. 3A). Amplification yielded fragments of 388, 586, 220, and 418 bp (Fig. 3B, lanes 6–9) that were consistent with the predicted sizes for the full-length PRL mRNA and similar to those amplified by the same primer combinations in the rat PRL cDNA positive control (Fig. 3B, lanes 2–5). No positive signal was detected in the absence of reverse transcriptase (Fig. 3B, lane 10) or in the negative control without RNA (Fig. 3B, lane 1). Expression of PRL mRNA in RRCECs was confirmed by *in situ* hybridization, using an antisense PRL RNA probe that positively labeled the perinuclear and/or nuclear areas of more than 90% of cells (Fig.

3C). Specificity of PRL mRNA expression was confirmed by the absence of a positive reaction with the sense riboprobe (not shown).

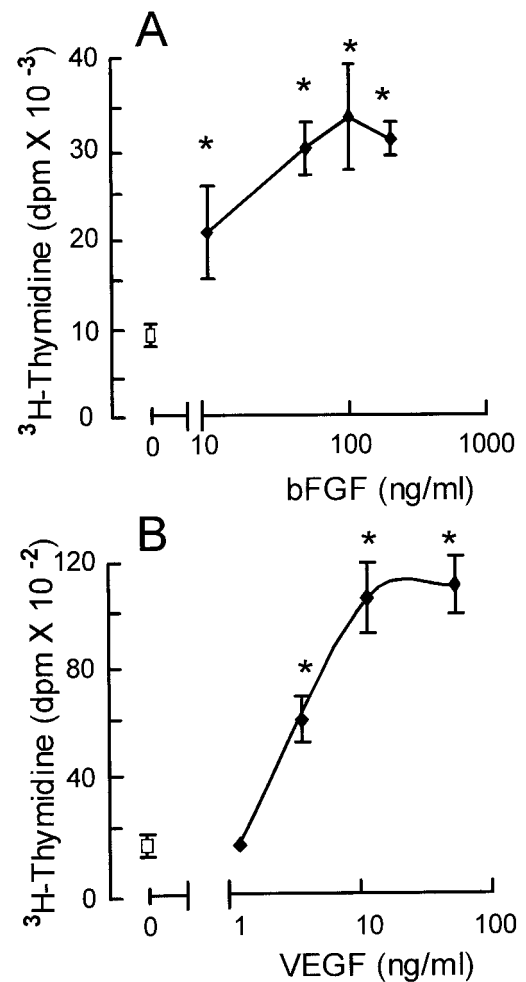


FIGURE 2. bFGF- and VEGF-induced proliferation of RRCECs. RRCECs proliferated in response to increasing concentrations of bFGF (A) and the specific endothelial cell mitogen VEGF (B). Data are mean \pm SEM of triplicate determinations. **P* < 0.05 versus basal proliferation.

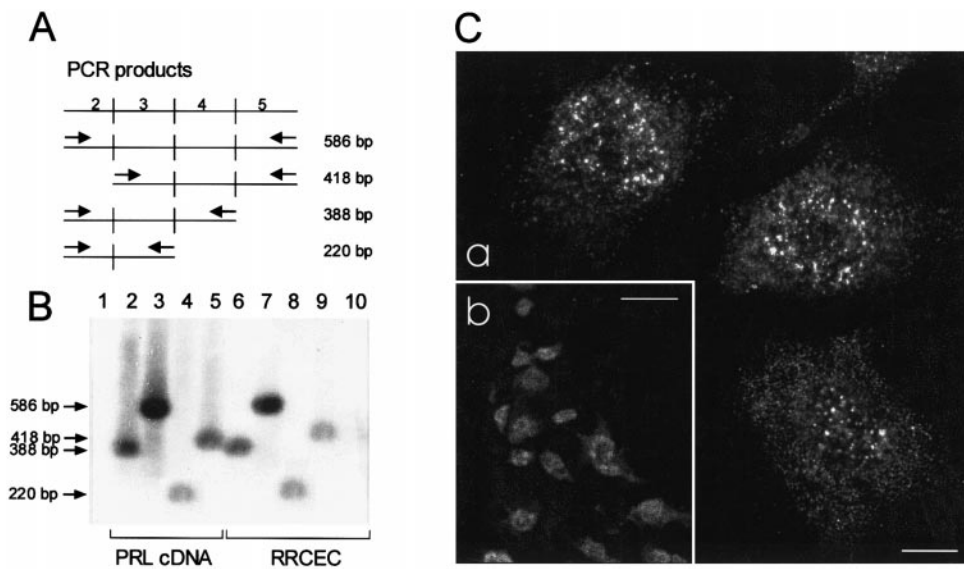


FIGURE 3. Expression of PRL mRNA in RRCECs. (A) Schematic representation of expected PCR products using primers (*arrows*) complementary to exons 2 to 5 of the PRL gene. The predicted sizes of PCR products for each primer combination are given in base pairs. (B) Southern blot analysis of PCR products from reverse transcribed total RNA from RRCECs (*lanes 6–9*) amplified (30 cycles) with the four combinations of PRL primers shown in (A). Similar PCR products were amplified from PRL cDNA (*lanes 2–5*). Negative controls were without RNA (*lane 1*) and without reverse transcriptase (*lane 10*). (C) Presence of the PRL mRNA in RRCECs as revealed by in situ hybridization and confocal microscopy. Scale bar, (Ca) 6 μm ; (Cb) 30 μm .

Expression of PRL Protein by RRCECs

Anti-PRL antiserum coupled to light immunofluorescence labeled the cytoplasm of more than 90% of RRCECs (Fig. 4A). Specificity of immunostain was ascertained by its neutralization with 1 μM PRL and by the absence of reaction to normal rabbit serum (not shown).

Western blot analysis probed with anti-PRL antiserum revealed a 23-kDa immunoreactive protein in both RRCEC lysates and conditioned media (Fig. 4B). Specificity of antibody reaction was indicated by absence of protein after PRL antiserum was preabsorbed with 1 μM PRL (Fig. 4B) or after blots were probed with normal rabbit serum (not shown). ELISA determinations showed that the levels of immunoreactive PRL in media conditioned for 24 hours with RRCECs were two times those in cellular lysates (Fig. 4C).

In support of the PRL nature of the 23-kDa immunoreactive protein, RRCEC-conditioned media, but not nonconditioned media, stimulated the proliferation of Nb2 cells in a dose-dependent manner (Fig. 5B). Stimulation by conditioned media and by PRL standard was abolished by PRL antiserum (Fig. 5A). The level of activity in the conditioned media of RRCECs was equivalent to 10 ng/ml of 23-kDa PRL, as estimated by serial dose-response effects of the rat 23-kDa PRL standard and after correcting values for a 10 \times concentration factor. Accordingly, both the bioassay and the ELISA measured equivalent PRL levels in RRCEC-conditioned media.

PRL Effects on RRCEC Proliferation

To investigate whether PRL is active on RRCECs, we tested the effect of 23-kDa and 16-kDa PRLs on RRCEC proliferation. Whereas 16-kDa PRL inhibited in a dose-dependent fashion the proliferation of RRCECs induced by bFGF, no effect followed treatment with 23-kDa PRL (Fig. 6A). Similarly, rat 23-kDa PRL, human 23-kDa PRL, and lactogenic human growth hormone did not affect basal proliferation of RRCECs (Fig. 6B). Consistent with the absence of effect, no evidence for the expression of the cloned PRL receptor could be obtained through RT-PCR in which primer combinations were used that were designed to amplify the long, medium, or short forms of the PRL receptor (Fig. 7, lane 1). Conversely, amplification of total RNA isolated from whole rat retinas by using the same primers yielded a 588-bp transcript (Fig. 7, lanes 3–6). The size of this transcript is consistent with the one predicted for the PRL receptor mRNA and is similar to that of products amplified by

the same primer combination in Nb2 cells (Fig. 7, lane 2), hypothalamus (Fig. 7, lane 8), and the rat PRL receptor cDNA (Fig. 7, lane 9), positive controls.

DISCUSSION

Endothelial cells play critical roles in a large number of physiologic and pathologic processes, such as leukocyte trafficking, inflammation, wound healing, tumor metastasis, and angiogenesis. The role of endothelial cells in these events varies between macrovascular and microvascular endothelium and is known to be affected by the anatomic location of the vascular bed.^{40,41} In the present study, endothelial cells from the microcirculation of the retina produced and released PRL, a pleiotropic protein with effects on reproduction, osmoregulation, immunomodulation, and angiogenesis.¹⁸

Expression of PRL mRNA in RRCECs was demonstrated by the RT-PCR amplification of PRL transcripts of the size expected for the full-length PRL mRNA encoding a 23-kDa PRL, the predominant PRL isoform. Similarly, the expression of the PRL mRNA was confirmed in RRCECs by in situ hybridization. In addition, fluorescence immunocytochemistry and Western blot analysis provided evidence for the translation of PRL mRNA in RRCECs. Accordingly, the cytoplasm of RRCECs contained PRL-like antigens that associated with a 23-kDa PRL-like immunoreactive protein present in both RRCEC lysates and conditioned media. Because of its apparent molecular weight, the 23-kDa protein could correspond to native unmodified PRL. Altogether, these results indicate that retinal endothelium expresses the PRL gene and the major 23-kDa PRL isoform.

Consistent with the release of 23-kDa PRL by retinal endothelial cells, RRCEC-conditioned medium stimulated the proliferation of Nb2 cells. Mitogenesis of the pre-T rat lymphoma Nb2 cells is dependent on lactogenic hormones,⁴² and 23-kDa PRL is the ligand known to activate signal transduction by the PRL receptor in these cells.⁴³ In addition, the bioassay and the ELISA measured equivalent PRL concentrations in RRCEC-conditioned media. Because both assays were standardized using 23-kDa PRL (NHPP standard), the equivalent PRL values measured by the two assays further indicate that the PRL-like protein in RRCEC-conditioned media corresponds to 23-kDa PRL.

Expression of the PRL gene in RRCECs confirms previous observations in endothelial cells from bovine brain capillaries

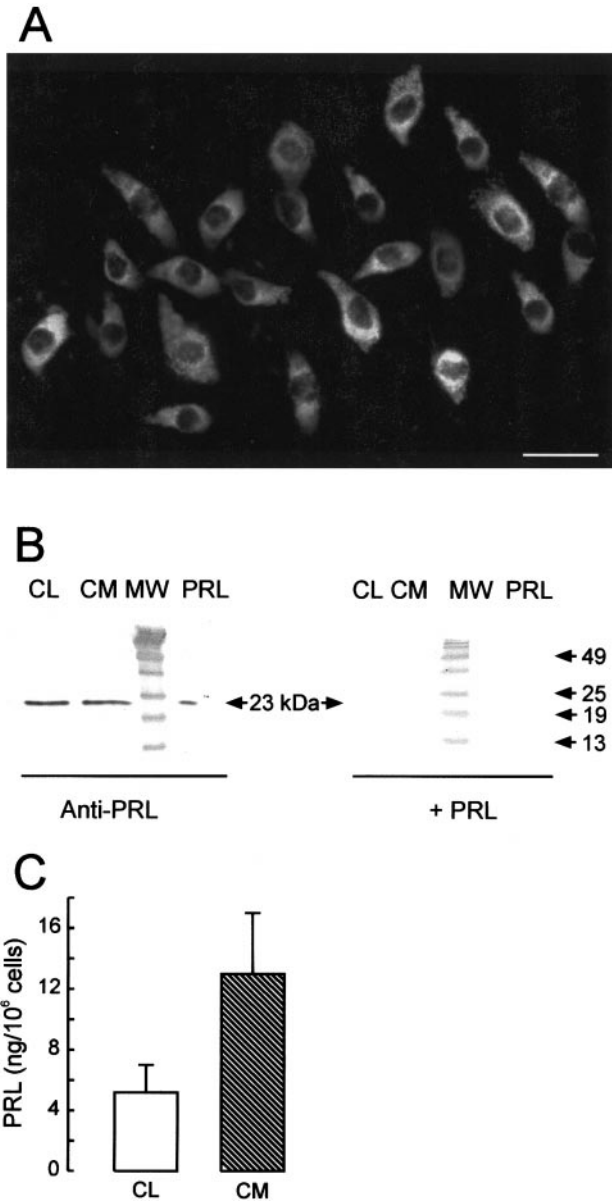


FIGURE 4. Expression of PRL in RRCECs. (A) PRL expression was detected in more than 90% of RRCECs after fluorescence immunocytochemistry with anti-PRL antibodies. (B) Western blot analysis probed with anti-PRL antibodies showing a 23-kDa immunoreactive protein in cell lysates (CL) and conditioned medium (CM) from RRCECs. Specificity of antibody reaction was confirmed by absence of positive signal after preabsorption of anti-PRL antibodies with 1 μ M PRL. (C) ELISA determination of PRL in CL and CM of RRCECs cultured for 24 hours. Data are mean \pm SEM of three independent experiments. MW, molecular weight standard. Scale bar, 30 μ m.

and human umbilical veins.^{27,28} However, dissimilarities in the type of PRL mRNA and protein expressed were noted between RRCECs and the other endothelial cells. Whereas RRCECs transcribed only the full-length PRL message and produced only the 23-kDa PRL isoform, the other endothelial cells express PRL mRNAs of different sizes and synthesize PRLs of 23, 21, 16, and 14 kDa.^{27,28} In addition, RRCECs released more than 300 times the amount of bioactive PRL estimated to be secreted by endothelial cells from bovine brain capillaries (30 pg/ml),²⁷ or from human veins where PRL levels are too low to be quantitated.²⁸

These differences in PRL production and secretion between rat retina and bovine brain and human umbilical cells illustrate the functional heterogeneity of endothelial cells. Functional dissimilarities between various endothelial cell types, including retinal endothelium, have been postulated both *in vivo* and *in vitro*.⁴¹ For example, retinal endothelial cells are known to maintain *in vitro* some of their distinct characteristics associated with their *in vivo* blood-retinal barrier function⁴⁴ and stand among other endothelial cell subtypes in their ability to express VEGF under basal conditions.⁴⁵

The functional implication of the PRL phenotype of retinal endothelium—that is, production and release of high levels of PRL, is unknown. Retinal endothelial cells may function as an ocular source for PRL, although its relation to PRL detected in the aqueous humor of rats²⁵ and in the aqueous humor and subretinal fluid of patients with retinopathy of prematurity²⁶ is unclear. PRL acts as a hormone or cytokine on functions that range from reproduction and osmoregulation to immunomodulation.

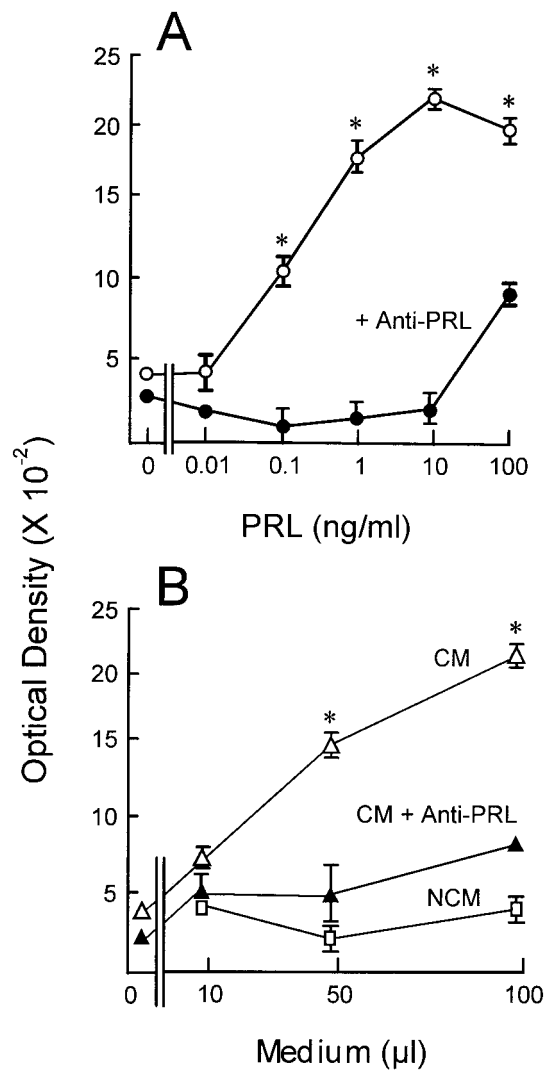


FIGURE 5. Secretion of bioactive PRL by rat RRCECs. (A) Proliferation of Nb2 cells in response to PRL (NIH standard) alone or together with anti-PRL antibodies. (B) Proliferation of Nb2 cells in response to RRCEC-conditioned medium (CM) was blocked by anti-PRL antibodies to levels similar to those induced by nonconditioned medium (NCM). CM and NCM were concentrated 10-fold. Proliferation was determined by a colorimetric assay, followed by optical density measurement at 595 nm. Data are mean \pm SEM of triplicate determinations. **P* < 0.05 versus cells without PRL or CM.

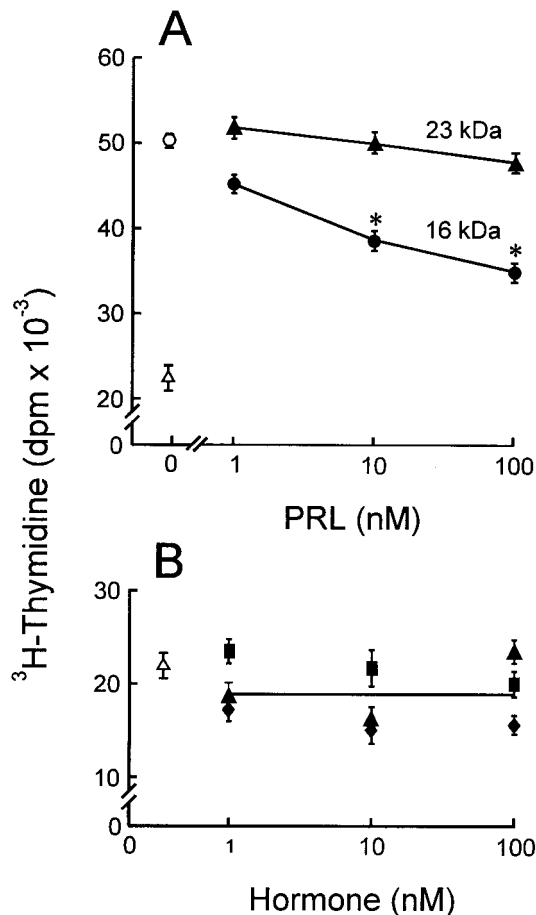


FIGURE 6. Modulation of RRCEC proliferation by 16-kDa but not by 23-kDa PRL. (A) Effect of 16-kDa and 23-kDa PRL on bFGF-induced proliferation of RRCECs. RRCECs were cultured in the absence (Δ) or presence (\circ) of bFGF alone or together with increasing concentrations of rat 16-kDa PRL (\bullet) and rat 23-kDa PRL (\blacktriangle). (B) Absence of effect of increasing concentrations of rat 23-kDa PRL (\blacktriangle), human 23-kDa PRL (\blacksquare), and human growth hormone (\blacklozenge) on RRCEC basal proliferation. Data are mean \pm SEM of triplicate determinations. * $P < 0.05$ versus bFGF alone.

lation and angiogenesis.¹⁸ Although antiangiogenic effects of PRL fragments are well documented,^{16,17,20-24} the effects of 23-kDa PRL on angiogenesis are controversial. The 23-kDa PRL appears to stimulate neovascularization in late, but not in early, stages of formation of the chick chorioallantoic membrane.^{16,46} Moreover, in vitro studies that failed to show 23-kDa PRL's effects and PRL receptor expression in endothelial cells from different vessels and species^{16,21,23} have been counteracted by a recent study showing that 23-kDa PRL can alter

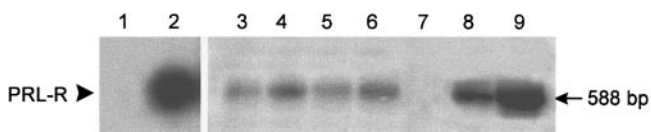


FIGURE 7. Expression of PRL receptor mRNA in whole rat retinas. Southern blot analysis of PCR products using primers complementary to the three forms of the rat PRL receptor cDNA. A 588-bp transcript was amplified from reverse-transcribed total RNA from four different rat retinas (lanes 3-6) but not from rat retinal capillary endothelial cells (lane 1). The 588-bp transcript was also amplified from Nb2 cells (lane 2), rat hypothalamus (lane 8), and PRL receptor cDNA (lane 9) as positive controls. Negative control was without RNA (lane 7).

the actin cytoskeleton and adhesion properties of injured pulmonary artery endothelial cells and that these cells express the PRL receptor.⁴⁷ In this regard, the present work argues against direct effects of 23-kDa PRL on retinal endothelium, because no evidence was obtained for the expression of any of the known PRL receptor isoforms in RRCECs, and 23-kDa PRL did not modify bFGF-induced proliferation or basal proliferation of these cells.

Nevertheless, 23-kDa PRL could affect endothelial cells indirectly by acting as the molecular precursor of fragments with antiangiogenic actions. In this regard, 16-kDa PRL inhibited bFGF-induced RRCEC proliferation, and recent evidence has suggested that antiangiogenic PRL fragments are present in ocular tissues, such as the cornea. The 16-kDa PRL inhibits bFGF-induced corneal neovascularization, and implants containing anti-PRL antibodies induce a local angiogenic reaction in the cornea.²⁴ Moreover, PRL can be cleaved into 16-kDa PRL by vitreous proteases and 16-kDa PRL can be detected in retinal homogenates (Dueñas and Clapp, unpublished observations, 2000).

However, endothelium-derived PRL may act as a paracrine regulator of retinal cells. Our results show the expression of the PRL receptor mRNA in the retina, and early studies have provided evidence for PRL's effects on the retina. These include putative effects on the metamorphosis of visual pigments in amphibians⁴⁸ and the regulation of thyrotropin-releasing hormone receptors⁴⁹ and photoreceptor destruction⁵⁰ in rats.

In this study endothelial cells from the microcirculation of the retina actively produced and released PRL. Identification of its functional role and proteolytic processing by ocular tissues warrants further investigation.

Acknowledgments

The authors thank Fernando López-Barrera, Olivia Vázquez, and Pilar Galarza for expert technical assistance.

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APENDICE 3

Prolactin in Eyes of Patients with Retinopathy of Prematurity: Implications for Vascular Regression

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PURPOSE. Disruption of the anti-angiogenic environment of the retina leads to neovascular eye diseases, including retinopathy of prematurity (ROP). Prolactin (PRL), the hormone originally associated with milk secretion, is proteolytically processed to 16K-PRL, a fragment with potent antiangiogenic, proapoptotic effects. Whether 16K-PRL is produced in eyes of patients with ROP and promotes the regression of intraocular blood vessels associated with this disease was investigated.

METHODS. PRL was quantified in the aqueous humor, subretinal fluid, and serum from patients with stage 5 ROP and in patients with non-neovascular eye disorders. Intraocular expression of PRL was evaluated by RT-PCR, in situ hybridization, and Western blot analysis. AntiPRL antibodies were injected intravitreally in neonatal rats, and apoptosis of hyaloid vessels determined by TUNEL and ELISA.

RESULTS. PRL was elevated in ocular fluids and serum from ROP patients. There was no correlation between PRL in ocular fluids and its level in serum, whereas PRL in aqueous humor and subretinal fluid were significantly correlated. PRL mRNA was expressed in blood vessels and leukocytes within retrolental fibrovascular membranes of ROP patients, and these membranes contained a 16 kDa immunoreactive PRL. The 16K-PRL isoform was more concentrated in subretinal fluid than in serum and was generated from PRL by subretinal fluid proteases. Intravitreal injection of neutralizing antiPRL antibodies inhibited the apoptosis of hyaloid vessels in neonatal rats.

CONCLUSIONS. 16K-PRL derived from PRL internalized from the circulation or synthesized intraocularly can stimulate apoptosis-induced vascular regression and contribute to the development and progression of ROP. (*Invest Ophthalmol Vis Sci.* 2004;45:2049-2055) DOI:10.1167/iovs.03-1346

Retinopathy of prematurity (ROP) is a major cause of blindness in children throughout the world.¹ Current treatment damages the retina and is frequently only partially effective at reducing blindness. ROP is initiated by delayed retinal vascular growth after premature birth, which leads to hypoxia-induced formation of surplus abnormal vessels and the attendant risk of retinal detachment and blindness.^{1,2} These alterations reflect an imbalance between factors that either stimulate or inhibit vessel growth. Vasoproliferative retinopathies are associated with elevated levels in the eye of multiple inducers of angiogenesis, including vascular endothelial growth factor,^{3,4} basic fibroblast growth factor (bFGF),⁵ insulin-like growth factor -1,⁶ angiogenin,⁷ placental growth factor,⁸ and hepatocyte growth factor.⁹ Likewise, pigment epithelium-derived factor, a major inhibitor of angiogenesis in the eye, is downregulated during ocular neovascularization.¹⁰ However, in contrast to angiogenesis stimulators, few angiogenesis inhibitors are known to operate in the eye, and little attention has been given to the discovery of new inhibitors.¹¹ This is surprising, considering that ocular tissues are maintained physiologically without the occurrence of neovascularization, and the ocular vasculature is highly restricted despite the presence of many angiogenesis stimulators in the eye.^{5,12,13}

PRL, originally identified as a lactotrophic hormone secreted by the pituitary gland, is now known to be produced by numerous extrapituitary tissues, including endothelial cells,¹⁴⁻¹⁶ neuronal, and immune cells,¹⁷ and it is implicated in a vast array of physiological functions that range from reproduction and osmoregulation to immunomodulation and angiogenesis.^{18,19} PRL can be proteolytically cleaved to 16K-PRL, a fragment that acts as a potent inhibitor of angiogenesis both in vivo and in vitro, inhibiting endothelial cell proliferation,²⁰ and stimulating expression of the type-1 plasminogen activator inhibitor²¹ and endothelial cell apoptosis.²² The potential involvement of 16K-PRL in ocular angiogenesis is suggested by studies showing that 16K-PRL inhibits bFGF-induced corneal angiogenesis, and that implants containing antiPRL antibodies induce angiogenesis in the cornea.²³ In addition, PRL mRNA and PRL and 16K-PRL have been detected in the cornea, iris, and retina of rats,²⁴ and cultures of rat retinal capillary endothelial cells express and release PRL.¹⁶ Furthermore, hypoxia, the main trigger of ocular neovascularization, decreases PRL synthesis and suppresses its conversion to 16K-PRL in rat pituitary tumor cells.²⁵ Here, measurements were made of PRL and 16K-PRL in sera, ocular fluids and fibrovascular membranes

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Supported by National Autonomous University of Mexico Grants PUIS, and IN227502, the National Council of Science and Technology of Mexico (CONACYT) Grant 36041-N, and Howard Hughes Medical Institute Grant 55000595.

Submitted for publication December 12, 2003; revised February 12, 2004; accepted February 25, 2004.

Disclosure: **Z. Dueñas**, None; **J.C. Rivera**, None; **H. Quiróz-Mercado**, None; **J. Aranda**, None; **Y. Macotela**, None; **P. Montes de Oca**, None; **F. López-Barrera**, None; **G. Nava**, None; **J.L. Guerrero**, None; **A. Suarez**, None; **M. De Regil**, None; **G. Martínez de la Escalera**, None; **C. Clapp**, None

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from patients with ROP. Evidence is presented that they are synthesized within the eye and that 16K-PRL can promote vascular regression in ROP. A preliminary report on some of these findings has appeared (Quiroz-Mercado H, et al. *IOVS* 2000;41:ARVO Abstract 1766).

METHODS

Study Subjects

Undiluted samples of aqueous humor, subretinal fluid, serum, and retrolental fibrovascular membrane (FVM) were obtained from patients with stage 5 ROP undergoing open-sky vitrectomy. For comparison, PRL determinations in aqueous humor and serum were taken from age-matched infants undergoing intraocular surgery for congenital cataracts. The protocol for sample collection followed the tenets of the Declaration of Helsinki, was approved by the Ethics Committee of the Hospital "Luis Sánchez Bulnes," and informed consent was obtained from the infants' parents. Age at surgery ranged from 6 months to 3 years. Medical histories were taken of these patients to exclude any underlying systemic disease including diabetes mellitus, congestive heart failure, hypertension, renal or hepatic insufficiency, and seizure disorders. The duration and extent of retinal detachment were recorded. Blood samples were obtained from the anesthetized infants immediately before surgery, and sera were immediately separated by 5 minutes microfuge centrifugation, and stored at -70°C until assayed.

Enzyme-Linked Immunosorbent Assay (ELISA) kit for PRL was purchased from Alexon-Trend Laboratories (Ramsey, MN) and used according to instructions with a detection limit of 2 ng/mL.

Bioassay

Bioactive PRL was determined using the Nb2-cell bioassay as detailed previously.²⁶ Incubations were carried out for 48 hours in the absence or presence of different dilutions of aqueous humor, subretinal fluid, serum, or of the human PRL standard purchased from A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA) with or without a 1:500 dilution of PRL antiserum. The human PRL antiserum (HC-1) was generated in our laboratory and characterized as described.¹⁵ Proliferation of Nb2 cells is linear in the range of 0.05 to 1 ng/mL PRL and is a standard procedure used to determine PRL levels in serum samples.²⁷

Immunoprecipitation–Western Blot Analysis

Size heterogeneity of PRL was determined in subretinal fluid and serum samples by immunoprecipitation–Western blot analysis using antiPRL antiserum (HC-1), and the previously reported technique.¹⁵ Optical density values were determined using 1D image analysis software, version 3.5 (Eastman Kodak Company, Rochester, NY).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA from FVM of ROP patients was obtained and RT-PCR performed essentially as described by Clapp and colleagues.²⁸ Two primers complementary to human PRL cDNA were synthesized: upstream primer from exon 2 (5'-GATGCCAGGTGACCCTTCGAGA-3') and downstream primer from exon 5 (5'-GCAGTTGTGTTGTGGATGATT-3'). RT-PCR products were confirmed by Southern blot analysis.

In Situ Hybridization

Sense and antisense PRL mRNA probes were transcribed in vitro from a linearized plasmid (pcDNA3; Invitrogen, Carlsbad, CA) containing the cDNA for human PRL with T7 and SP6 polymerases and labeled with Digoxigenin-UTP (Boehringer Mannheim, Mannheim, Germany). FVM from ROP patients were washed in PBS, embedded in Tissue-Freezing Medium (Leica Instruments, Nussloch, Germany), sectioned (10 μM), and subjected to in situ hybridization performed as previously described.¹⁶

PRL Cleavage Analysis

The activity of the enzymes that cleave PRL to 16K-PRL was assayed by the reported method²⁹ with the following modifications. Briefly, 10 μL of subretinal fluid diluted 1:5 in water were mixed with 10 μL of the human PRL standard (20 ng per μL of 0.1 M Tris-HCl, pH 7.4) and with 20 μL of reaction buffer (0.1 M citrate-phosphate, 0.15 M NaCl, pH 5.0) for 24 h at 37°C . The PRL cleavage products were separated by SDS-PAGE under reducing conditions and subjected to Western blot analysis.

Intravitreal Injection

An antirat PRL polyclonal antibody able to neutralize the activity of PRL and 16K-PRL in vivo²³ and in vitro was used.^{16,30} Animals were maintained and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wistar rats at postnatal day 10 (P10) were anesthetized with intraperitoneal pentobarbital (20 ng per g of body weight) and their eyes intravitreally injected with 2 μL (2 μg) of purified antiPRL polyclonal antibodies, control antibodies (purified from normal serum), or vehicle only (PBS). At P13, eyes were enucleated and the hyaloid tissue removed.

Apoptosis Determination

Samples were evaluated for evidence of DNA fragmentation associated with apoptosis by the following techniques.

TUNEL assay. FVM from ROP patients or the hyaloid tissue from rats was fixed in 4% paraformaldehyde in PBS, pH 7.4 for 10 minutes or 20 minutes, respectively. Subsequently, the tissues were washed in PBS, embedded in Tissue-Tek, sectioned (10 μm), and analyzed for apoptotic cells by terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) staining, using a Detection Kit (Roche Diagnostics, Mannheim, Germany) and a fluorescence microscope (Olympus BX60, Lake Success, NY). Each section was visually scanned with a high power (40X) objective in a serpentine manner to record the total number of TUNEL positive cells in the entire section. The total number of TUNEL-positive cells per unit area was calculated after determining the area by tracing the outline of each section with an image analysis system attached to the microscope.

ELISA. The apoptotic cell death detection ELISA (Boehringer Mannheim, Indianapolis, IN) was used to quantitatively determine fragmented nucleosomal DNA associated with apoptotic cell death, according to manufacturer's instructions.

Statistics

Experiments were replicated at least three times. Means were compared using Student's *t*-test, and correlations were evaluated by linear regression analysis. When comparing more than three groups, ANOVA with post hoc analysis was used. The significance level was set to 5%.

RESULTS

PRL Levels in Ocular Fluids and Serum from Patients with ROP

Samples were obtained from 48 patients (21 females and 27 males) with stage 5 ROP. The mean age of the patients was 1.2 years (range, 0.5 to 3 years). The ELISA measured PRL in all samples of subretinal fluid and serum with mean values of 23.3 ± 1.9 ng/mL and 43.0 ± 5.6 ng/mL, respectively (Fig. 1A). The concentration of PRL in serum was significantly higher ($P < 0.05$) than in subretinal fluid. In the aqueous humor, average immunoreactive PRL (4.8 ± 1.1 ng/mL) was lower ($P < 0.05$) than in the subretinal fluid, and was detected only in 51.3% of the samples (19 of 37 patients) (Fig. 1A). There was no significant correlation between PRL concentration and age ($P = 0.3$, ANOVA) or sex ($P = 0.4$, $P = 0.2$, and $P = 0.3$, for the aqueous humor, subretinal fluid, and serum, respectively).

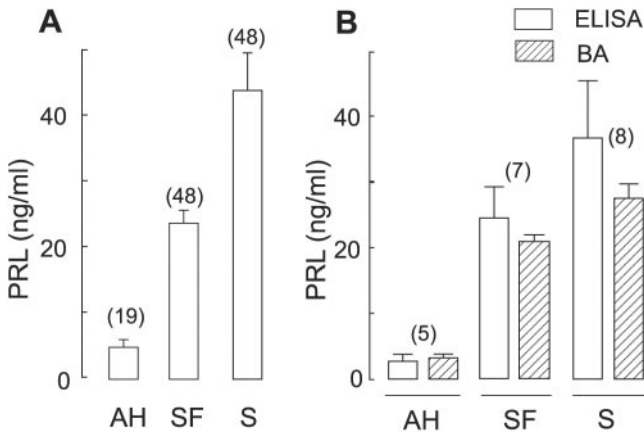


FIGURE 1. (A) PRL levels in the aqueous humor (AH), subretinal fluid (SF), and serum (S) of patients with retinopathy of prematurity determined by enzyme-linked immunosorbent assay (ELISA). (B) PRL levels in patients with ROP determined in the same samples by ELISA and by the specific PRL Nb2-cell bioassay (BA). The number of patients analyzed in each group is indicated in parentheses. Bars show the mean \pm SEM.

of the patients. To further substantiate the presence of PRL in ocular fluids, PRL concentration was determined in the same samples from a subgroup of ROP patients, by both ELISA and the specific PRL Nb2-cell bioassay (Fig. 1B). Both methods measured equivalent amounts of PRL. Because the sensitivity of the bioassay is 40-fold higher than that of ELISA, its use helped validate PRL concentrations in the aqueous humor, and confirmed the presence of PRL in ocular samples of ROP patients.

Elevation of PRL Levels in ROP Patients

PRL levels in ROP patients were compared to those in 18 patients (6 females and 12 males) with a non-neovascular eye disorder (congenital cataracts). The mean age of these patients was 3 years (range, 1 to 6 years). Because there is no retinal detachment in these patients, the subretinal fluid is not amenable to sampling. As in the ROP group, patients with congenital cataracts showed no correlation between PRL levels and the age or sex status. The mean PRL values measured in both the aqueous humor and serum of these patients were significantly lower than corresponding values in ROP patients (Fig. 2).

Lack of Correlation between Ocular and Circulating PRL Levels

To investigate whether ocular PRL could originate from systemic PRL, the concentrations of PRL in ocular fluids and those in serum were compared in the same individuals. However, no correlation was found between PRL concentration in aqueous humor and PRL concentration in serum ($r = 0.0103$, $P < 0.96$, Fig. 3), nor between PRL in subretinal fluid and PRL in serum ($r = 0.0993$, $P < 0.53$, Fig. 3). However, the concentration of PRL in aqueous humor was significantly correlated with its concentration in subretinal fluid ($r = 0.6791$, $P < 0.01$, Fig. 3).

PRL Synthesis in FVM of ROP Patients

RT-PCR—Southern blot analysis detected the expression of PRL mRNA in FVM from patients with ROP. RT-PCR amplified a single product with a corresponding length of 348 bp (Fig. 4A, lane 3), which is similar to the one amplified from the PRL cDNA positive control (Fig. 4A, lane 1). To localize the expression of the PRL message, FVM were subjected to in situ hybridization using an antisense PRL RNA probe. The membranes were comprised of a fibrous component with interspersed

blood vessels. PRL mRNA was detected in the vascular portions within vessel walls and in infiltrated leukocytes (Figs. 4B and 4D). In contrast, no apparent hybridization signal was found in FVM hybridized with the sense probe (Figs. 4C and 4E).

Intraocular Generation of 16K-PRL

Consistent with the translation of PRL mRNA and the posttranslational cleavage of the protein, a 16 kDa immunoreactive PRL was detected by Western blot analysis in lysates from FVM (Fig. 5A). In addition, immunoprecipitation–Western blot analysis revealed PRL immunoreactive proteins of 23 and 16 kDa in subretinal fluid and sera from three different ROP patients (Fig. 5B). The ratio of 16K-PRL to full-length protein (23K-PRL) was significantly higher ($P < 0.05$) in subretinal fluid than in serum from the same patients (1.0 ± 0.2 vs. 0.3 ± 0.08 , respectively). This difference may result from the local proteolysis of PRL by subretinal fluid proteases. To determine whether the proteolytic activity responsible for generation of 16K-PRL is present in subretinal fluid, exogenous PRL (200 ng) was incubated with 2 μ L of subretinal fluid, and PRL proteolysis was evaluated by Western blot analysis (Fig. 5C). Because the concentration of endogenous PRL in 2 μ L of subretinal fluid is not enough to be detected by Western blot analysis (Fig. 5C), any PRL-immunoreactive protein should derive from the exogenous hormone. After incubation, in addition to PRL, a second immunoreactive product was seen that corresponded in size to 16K-PRL (Fig. 5C). The generation of the 16 kDa product seemed to be attributable to the action of a protease in the subretinal fluid, because no 16K-PRL was evident when the 23K-PRL standard was incubated with heat-inactivated subretinal fluid or in the absence of subretinal fluid (Fig. 5C).

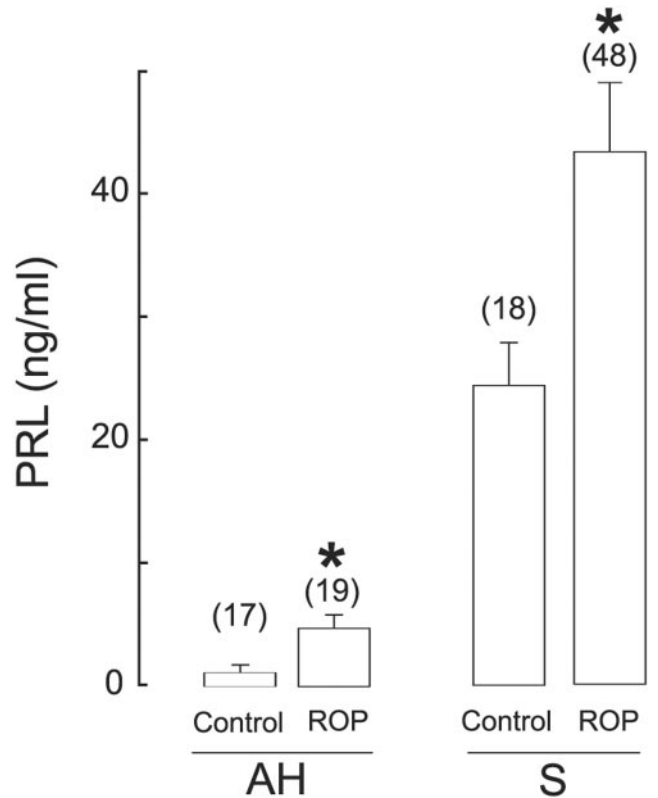


FIGURE 2. PRL levels in aqueous humor (AH) and serum (S) of patients with congenital cataracts (control) and retinopathy of prematurity (ROP) measured by ELISA. The number of patients analyzed in each group is indicated in parentheses. Bars show the mean \pm SEM; * $P < 0.05$ vs. values in control patients.

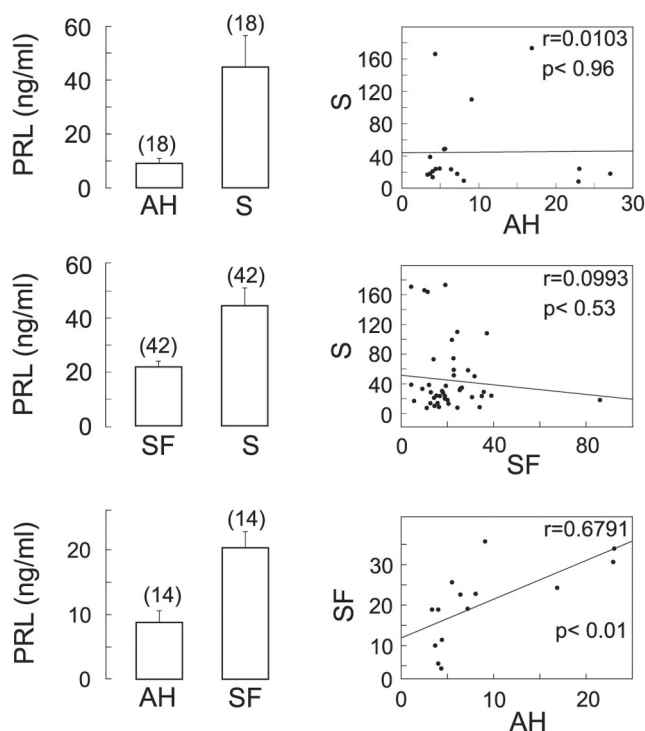


FIGURE 3. Lack of correlation between PRL levels in serum and PRL levels in aqueous humor (AH) or in subretinal fluid (SF), and significant positive correlation between PRL levels in AH and PRL levels in SF of patients with ROP. Determinations were by ELISA. The number of patients analyzed in each group is indicated in parentheses. Bars show the mean \pm SEM of the respective groups whose individual values were correlated. The correlation coefficient (r) and significance level (P) are indicated.

Apoptosis in FVM

Several TUNEL-positive cells were visualized throughout the FVM from ROP patients, many of which were associated with vascular structures (Figs. 6A, 6B, and 6C).

AntiPRL Antibodies Prevent Apoptosis in Hyaloid Vessels from Neonatal Rats

To investigate whether PRL-derived peptides could play a role in promoting apoptosis of blood vessels in FVM of ROP, evaluation was made of the effect of the intravitreal injection of neutralizing antiPRL antibodies on apoptosis of the hyaloid vascular system in neonatal rats. The hyaloid system is a transient network of intraocular vessels present during development; in humans it normally regresses by apoptosis before birth, whereas in the rat it regresses mainly between P10 and P20.³¹ We observed that at P13 the hyaloid tissue removed from rat eyes treated with control antibodies (Figs. 6D and 6E) or vehicle (not shown) showed prominent TUNEL staining in association with blood vessels and in cells interspersed within the stroma. In contrast, very few TUNEL-positive cells were detected in the hyaloid system from eyes injected with antiPRL antibodies (Figs. 6F and 6G). These differences were found to be statistically significant ($P < 0.01$) after counting the number of TUNEL-positive cells (Fig. 7A). Similarly, measurement of fragmented nucleosomal DNA by cell death ELISA showed that intravitreal administration of PRL antibodies significantly ($P < 0.01$) reduced apoptosis of the hyaloid tissue (Fig. 7B).

DISCUSSION

ROP is an ischemia-induced proliferative retinopathy that shares pathophysiological characteristics with other common ocular diseases including diabetic retinopathy and age-related macular degeneration. While different etiologies lead to insufficient perfusion of the retina, once a critical level of ischemia has been achieved, the different forms of retinopathy converge on the common hypoxia-induced formation of blood vessels that may invade the vitreous and lead to retinal detachment and blindness. Current laser therapy has reduced the incidence of blindness by suppressing neovascular growth leading to retinal detachment; however, visual outcome after treatment is often poor. Extending the knowledge of natural angiogenesis inhibitors within the eye is key to developing more effective approaches for the treatment and prevention of these diseases. 16K-PRL, a potent inhibitor of angiogenesis, is produced locally within the eye of patients with ROP, and may be involved in the regression of intraocular blood vessels.

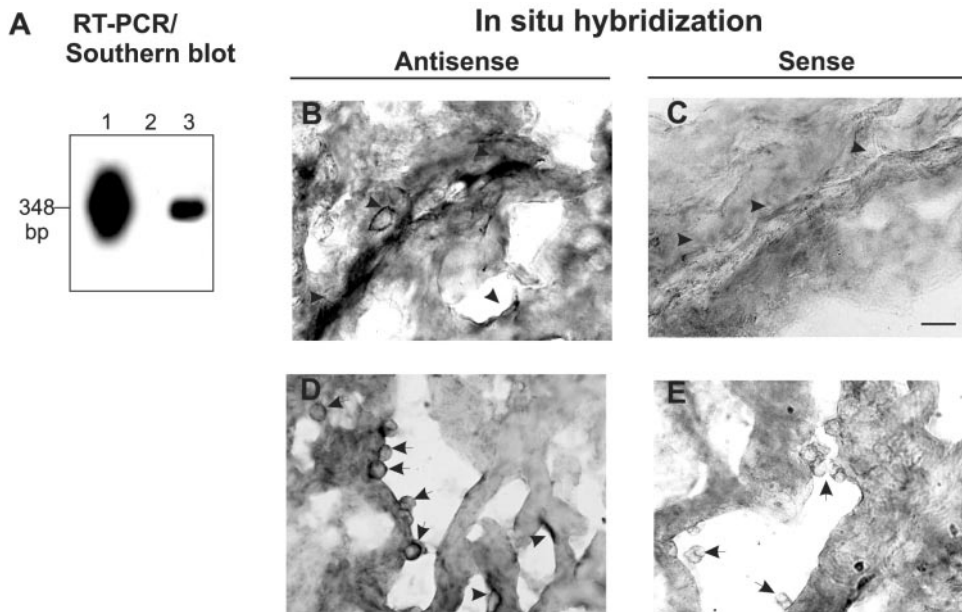
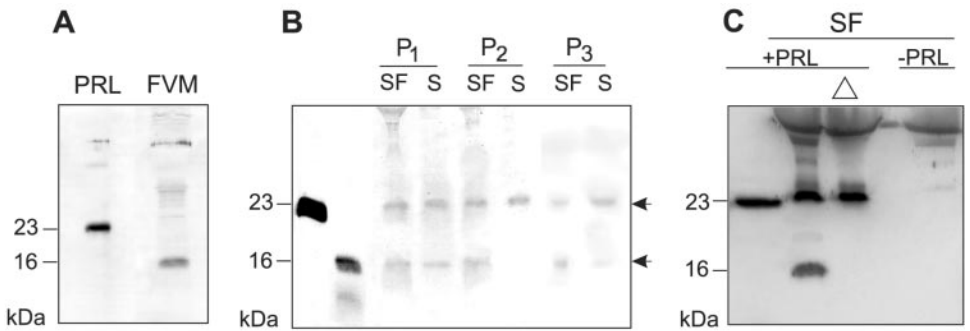


FIGURE 4. Expression of PRL mRNA in fibrovascular membranes of ROP patients. (A) Southern blot of the RT-PCR product obtained by using primers specific for PRL in fibrovascular membranes from patients with ROP (lane 3). PRL cDNA was used as a positive control (lane 1), and omission of reverse transcriptase served as a negative control (lane 2). (B) and (D) PRL mRNA is visualized by in situ hybridization with an antisense RNA probe in blood vessels (arrowheads) and in leukocytes (arrows) of fibrovascular membranes from patients with ROP. No positive signal follows hybridization with the sense probe (C, E). Scale bar = 50 μ m (B-E).

FIGURE 5. (A) Western blot analysis of immunoreactive PRL-like proteins in fibrovascular membranes (FVM) of patients with ROP. Human PRL standard marks the 23 kDa position. (B) Subretinal fluid (SF) and serum (S) samples from three different patients with ROP were immunoprecipitated and subjected to Western blot analysis. Immunoreactive proteins of 23 and 16 kDa are indicated (arrows). (C) Western blot analysis of PRL cleaved products generated after a purified human PRL standard was incubated in the absence (lane 1) or presence of subretinal fluid (SF) from patients with ROP, before (lane 2) and after heat inactivation for 30 minutes at 85°C (Δ , lane 3). SF incubated in the absence of PRL (lane 4). Blots are representative of three independent experiments.



PRL acts as a circulating hormone and as a cytokine in a wide variety of processes including angiogenesis. PRL can be posttranslationally modified by proteolytic cleavage to 16K-PRL, a fragment with antiangiogenic actions.¹⁹ PRL in the aqueous humor, subretinal fluid, and serum of patients with stage 5 ROP was determined by both ELISA and the specific Nb2-cell bioassay. The values did not vary with the gender or age of the patients, and were not influenced by emotional stress since samples were obtained from patients under general anesthesia. Actually, PRL values in serum of control patients (congenital cataracts) were equivalent to those reported in

anesthetized children (24.4 ± 3.4 vs. 18.4 ± 11.4 ng/mL, respectively) and in conscious children (13.3 to 25.5 ng/mL) with matching ages (between 1 and 6 years).^{32,33} Likewise, PRL levels in aqueous humor of control infants were similar to those reported in adult patients (0.5 to 1.9 ng/mL) undergoing cataract surgery.³⁴

Interestingly, higher PRL values were measured in serum and aqueous humor from ROP patients than from patients with a non-neovascular eye disorder (congenital cataracts). It is unclear whether this increase is functionally related to the disease. A functional connection between pituitary hormones and vasoproliferative retinopathies was hypothesized a long time ago after finding that regression of retinal neovascularization followed pituitary infarction.³⁵ In fact, based on this observation, pituitary ablation was used as a form of therapy for proliferative diabetic retinopathy.³⁶ Nevertheless, analysis of diabetics has either shown reduced circulating levels of PRL in association with severe retinopathy,³⁷ or no change in connection with this disease.³⁸ The reasons for these discrepancies are not immediately obvious, but may suggest that changes in systemic PRL relate to general conditions associated with the specific disease other than retinopathy itself. Diabetes can affect the secretion of PRL,³⁹ and several studies describe hyperprolactinemia in preterm and term infants.^{40,41}

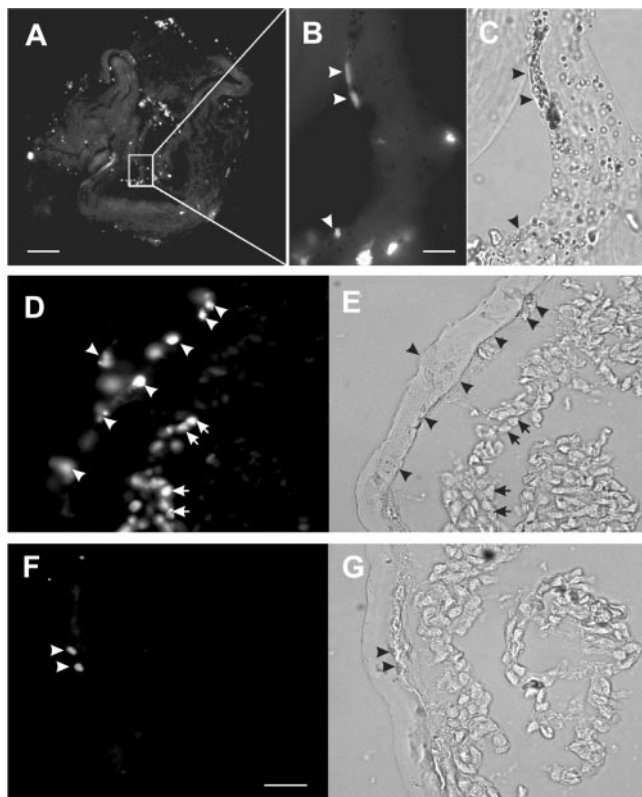


FIGURE 6. (A) TUNEL-positive cells in fibrovascular membranes from patients with ROP. Magnification of *insert* in (A) illustrates the association of TUNEL-positive cells with blood vessels (arrowheads) under fluorescence (B) and light-field microscopy (C). TUNEL-positive cells in the hyaloid vascular system of neonatal rats injected intravitreally with control antibodies (D) or antiPRL antibodies (F). Same fields illustrating TUNEL-labeled cells in blood vessels (arrowheads) under fluorescence (D, F) and light-field microscopy (E, G). Scale bar: (A) 400 μ m, (B, C) 50 μ m, and (D–G) 100 μ m.

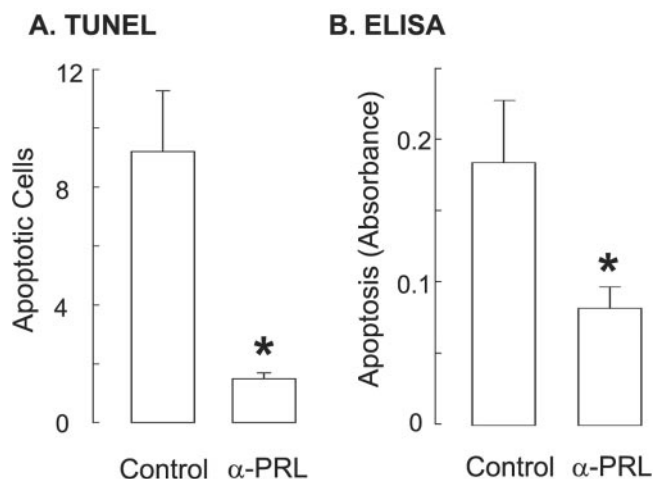


FIGURE 7. Quantitative analysis of apoptosis in the hyaloid system of the neonatal rat injected intravitreally with control antibodies (control) or with antiPRL antibodies (α -PRL). (A) The total number of TUNEL-positive cells was counted in each section and expressed per 50 μ m². (B) Apoptosis was quantified by cell-death ELISA. Absorbance was measured at 405 nm. Bars show the mean \pm SEM from three independent samples. * $P < 0.01$ vs. control.

High circulating PRL could cause an increase of its concentration in ocular fluids. Radioautographic studies have shown that iodinated PRL injected intracardially is incorporated into ocular tissues, including the retina, choroid, and ciliary body.⁴² Such incorporation could be mediated by specific receptor-mediated transport, since PRL receptors are localized in the ciliary epithelium (Dueñas Z and Clapp C, unpublished observations, 2003). Also, impairment of the blood ocular barrier in ROP patients⁴³ could favor ocular accessibility of the circulating hormone. However, against the latter, no correlation was found between the concentration of PRL in serum and PRL values in aqueous humor or in subretinal fluid of ROP patients. Nonetheless, the concentration of PRL in aqueous humor correlated significantly with that in subretinal fluid. This observation is consistent with subretinal fluid being derived mostly from the vitreous and the posterior aqueous humor flow,⁴⁴ and suggests that PRL is internalized into the eye by receptor-mediated transport at the level of the aqueous humor-producing ciliary epithelium.

In addition to its active uptake from the circulation, PRL can be synthesized intraocularly. PRL mRNA has been detected in the cornea, iris, and retina of rats,²⁴ and cultures of rat retinal capillary endothelial cells express and release PRL.¹⁶ Retrolental FVM of ROP patients express the PRL mRNA, localized within blood vessels and interspersed leukocytes. The cells expressing PRL in the blood vessels could be of endothelial origin, because endothelial cells from retinal capillaries¹⁶ and other vascular beds, in species including the human, synthesize PRL.^{14,15} Also, finding PRL mRNA in leukocytes is not surprising, since compelling evidence shows that immune cells express and respond to this hormone.¹⁷ Whereas the mRNA amplified by RT-PCR corresponded in size to the one encoding for the full-length protein (23K-PRL), Western blot analysis of homogenates from FVM only detected a 16 kDa PRL-like protein. Thus, in these membranes, as at other PRL-producing sites,¹⁹ 16K-PRL appears to be generated not by alternative splicing but by the proteolytic cleavage of PRL. The demonstration that subretinal fluid contains PRL, 16K-PRL, and PRL-cleaving enzymes, substantiates this conclusion. Also, the higher proportion of 16K-PRL found in subretinal fluid relative to that in serum provides evidence that the cleavage of PRL to 16K-PRL can take place intraocularly.

Whether derived from the cleavage of PRL internalized from the circulation or synthesized intraocularly, 16K-PRL may play an important role in the development and progression of ROP. 16K-PRL can halt angiogenesis by inducing the apoptosis of endothelial cells,²² and endothelial cell apoptosis is an important event mediating the regression of blood vessels in ROP, which in turn, can lead to the resolution of the disease.⁴⁵ Investigators have found that two thirds of infants who are born weighing 1250 g or less develop ROP, but only ~6% require treatment,⁴⁶ and that even severe ROP can undergo successful spontaneous involution.⁴⁷ This report demonstrates that blood vessels in FVM from stage 5 ROP are undergoing apoptosis-mediated regression. Because pro-apoptotic 16K-PRL is present in these membranes, 16K-PRL may promote vascular regression in ROP patients.

The vascular system in ROP membranes includes portions of the hyaloid vasculature,⁴⁸⁻⁵¹ a transient network of intraocular vessels that nourish the immature lens, retina, and vitreous. This system normally regresses before birth, but in premature infants hyaloid vessels can persist and exacerbate ROP.⁴⁸⁻⁵¹ In other mammals such as the rat, the hyaloid system remains after birth and regresses via apoptosis by the second week postpartum.³¹ To investigate whether 16K-PRL can promote the apoptosis of blood vessels in ROP, the contribution of ocular PRLs to apoptosis of hyaloid vessels in the neonatal rat was examined. Intravitreal administration of anti-PRL antibodies, but not of control antibodies, caused a significant

reduction in the number of hyaloid cells undergoing apoptosis, as measured by two independent methods. These findings are consistent with the antibodies sequestering endogenous PRLs able to promote apoptosis of hyaloid vessels, and thus, with PRL molecules having a role in retinal vascular development. Because the antibodies inhibit apoptosis of both vessel-associated and nonvessel-associated cells, it is clear that not only endothelial cells but also other cell types are targets of pro-apoptotic PRLs.

In summary, PRL levels are elevated in the circulation and in the eyes of ROP patients, suggesting that PRL-derived peptides promote the regression of retinal neovascularization in ROP. In these patients, high levels of ocular PRL, originating from internalized systemic PRL or from the hormone synthesized locally, may lead to an increase in the ocular concentration of 16K-PRL. This increase would help counterbalance pathologic angiogenesis by stimulating the apoptosis of blood vessels, and thus, contribute to the favorable resolution of the disease. In this regard, high levels of circulating PRL could have a favorable impact on the prognosis of ROP, since they may enable greater production of 16K-PRL within the eye. In addition, the finding that 16K-PRL promotes vascular regression is clinically relevant, indicating a potential treatment for patients who already have established ocular neovascularization. Notably, the observation that human milk feeding reduces ROP,⁵² can be explained on the basis of the present work, since high PRL concentrations are found in milk,⁵³ milk PRL can reach systemic circulation,⁵⁴ and PRL in systemic circulation can reach ocular fluids (present results). In conclusion, these findings link PRL peptides with the underlying causes of ROP, and with the prevention and course of the disease, and they warrant further investigation.

Acknowledgments

The authors thank María Antonieta Burgoa, Antonio Prado, Daniel Mondragón, Martín García, and Pilar Galarza for their expert technical assistance, and Dorothy D. Pless for editing the manuscript.

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