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Mechanismen immunologischer Toleranz

Untersuchungen am Plazenta- und Tumor-Modell

Habilitationsschrift

zum Erwerb der Venia Legendi

für das Fach

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vorgelegt von

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Für Julia

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Einleitung

Reproduktionsimmunologie

Ende des 18. Jahrhunderts wiesen die Gebrüder Hunter durch Wachsausguss nach, dass kindlicher und mütterlicher Kreislauf voneinander getrennt sind und nicht, wie bis dahin angenommen, ein kontinuierlicher Blutfluss von der Mutter zum Kind existiert. Die Austauschprozesse an der fetomaternalen Grenzzone sind seitdem Gegenstand des wissenschaftlichen Interesses. Der Immunologe und Nobelpreisträger Sir Peter Medawar richtete –ein Jahr vor der ersten geglückten Organtransplantation- 1953 den Blick auf das immunologische Paradox, dass der Fetus, obwohl er jeweils zur Hälfte mütterliche und väterliche Gene trägt, vom mütterlichen Immunsystem toleriert wird[13]. Dies widersprach grundlegend der damaligen Vorstellung der Selbst-Fremd-Erkennung und begründete die Forschungsrichtung der Reproduktionsimmunologie. Die Schwangerschaft ist damit ein natürliches, erfolgreiches Modell immunologischer Toleranz.

Beim Aufbau der fetomaternalen Grenzfläche wachsen fetale Zellen (sog. Trophoblasten) in die mütterliche Uterusschleimhaut ein, verschmelzen dabei miteinander, arrodieren mütterliche Blutgefäße und bilden in der reifen Plazenta die Auskleidung eines mütterlichen Blutsees[16]. Das trophoblastäre Synzytium ist also gleichermaßen fetales Epithel wie plazentares Endothel und interagiert mit mütterlichen Leukozyten[15].

Das mütterliche Immunsystem akzeptiert jedoch nicht einfach passiv das fetale Gewebe, sondern immunologische Mechanismen sind zentral an der Invasion des Trophoblasten in die mütterliche Dezidua beteiligt und formen die fetomaternale Grenzfläche. Dieses immunologische Milieu im Bereich der fetomaternalen Grenzzone trägt entscheidend zum Schwangerschaftserfolg bei.

Dem Immunsystem kommt in allen Organen die Aufgabe zu, einen physiologischen Grundzustand zu erhalten und im Falle einer Infektion das Pathogen zielgerichtet zu bekämpfen ohne jedoch das Organ selbst zu schädigen. Dies gilt auch für die fetomaternale Grenzzone. Immunologische Mechanismen sind hier jedoch so zentral in die plazentare Architektur und Entwicklung verwoben sind, dass eine manifeste Infektion oftmals eine

bleibende Schädigung der Plazentafunktion und damit eine Gefährdung des weiteren Schwangerschaftsverlaufs nach sich zieht.

Eine genauere Kenntnis der immunologischen Mechanismen im Bereich der fetomaternalen Grenzzone kann Hinweise auf die Pathogenese von Plazentaerkankungen liefern, die eine hohe perinatale Morbidität bedingen. Zu diesen Erkrankungen zählen die hypertensiven Schwangerschaftserkrankungen ebenso wie die fetomaternale Transfusion, aber auch die intrauterine Wachstumsretardierung und der intrauterine Fruchttod.

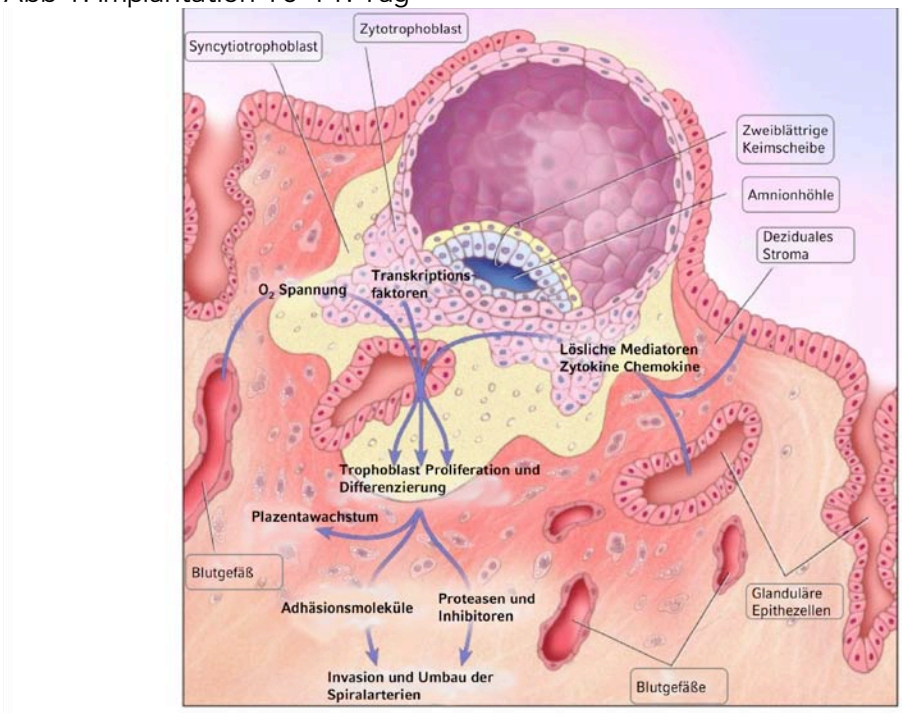
Die Frage immunologischer Toleranz ist auch in der Kanzerogenese und in der Etablierung des Tumormikromilieus von entscheidender Bedeutung[9]. Die Entstehung und immunologische Etablierung eines malignen Tumors ist die gemeinsame Endstrecke eines letztendlich ungerichteten Prozesses. Die Charakteristika einer malignen Erkrankung sind daher in hohem Maße individuell. Ausdruck dessen ist die zunehmende Hinwendung zu individualisierten Krebstherapien (sog. *targeted therapies*) wie sie z.B. auch immuntherapeutische Ansätze darstellen[8]. Der spezifische Aufbau immunologischer Toleranz an der Tumor-Stroma Grenzfläche ist auf Grund der großen interindividuellen Unterschiede im humanen System nur schwer nachzuvollziehen. Demgegenüber verläuft der Aufbau des spezifischen immunologischen Mikromilieus an der fetomaternalen Grenzfläche entlang geordneter Bahnen, deren Erforschung allgemeine Prinzipien der Toleranzentwicklung im humanen System zu Tage fördern könnte. Die vorliegende Habilitationsschrift widmet sich Mechanismen immunologischer Toleranz und ihrer Durchbrechung am Plazenta- und Tumor-Modell.

Plazentation

Plazentation als invasiver Prozess

Der kindliche Anteil der Plazenta, der Trophoblast, differenziert sich in zwei Schichten. Der Zytotrophoblast (ZT) umgibt am Beginn der Implantation die Blastozyste wie eine Schale. Der zuvorderst in die Uterusschleimhaut eindringende Anteil des Trophoblasten verliert seine Zellgrenzen und verschmilzt zu einem Synzytium (Synzytiotrophoblast, ST). Der ST dringt aktiv in das mütterliche Endometrium ein und löst Umbauprozesse aus, die zur Arrosion mütterlicher Kapillaren und zur Bildung blutgefüllter Lakunen führen. Damit hat der kindliche Anteil der Plazenta ca. ab dem 10. embryonalen Entwicklungstag Anschluss an das mütterliche Gefäßsystem gefunden. Die Grenzfläche und weitere Invasionsfront bildet dabei der Synzytiotrophoblast. Die Zytotrophoblastzellen stellen dessen Wachstumsreservoir dar.

Abb 1. Implantation 10-11. Tag

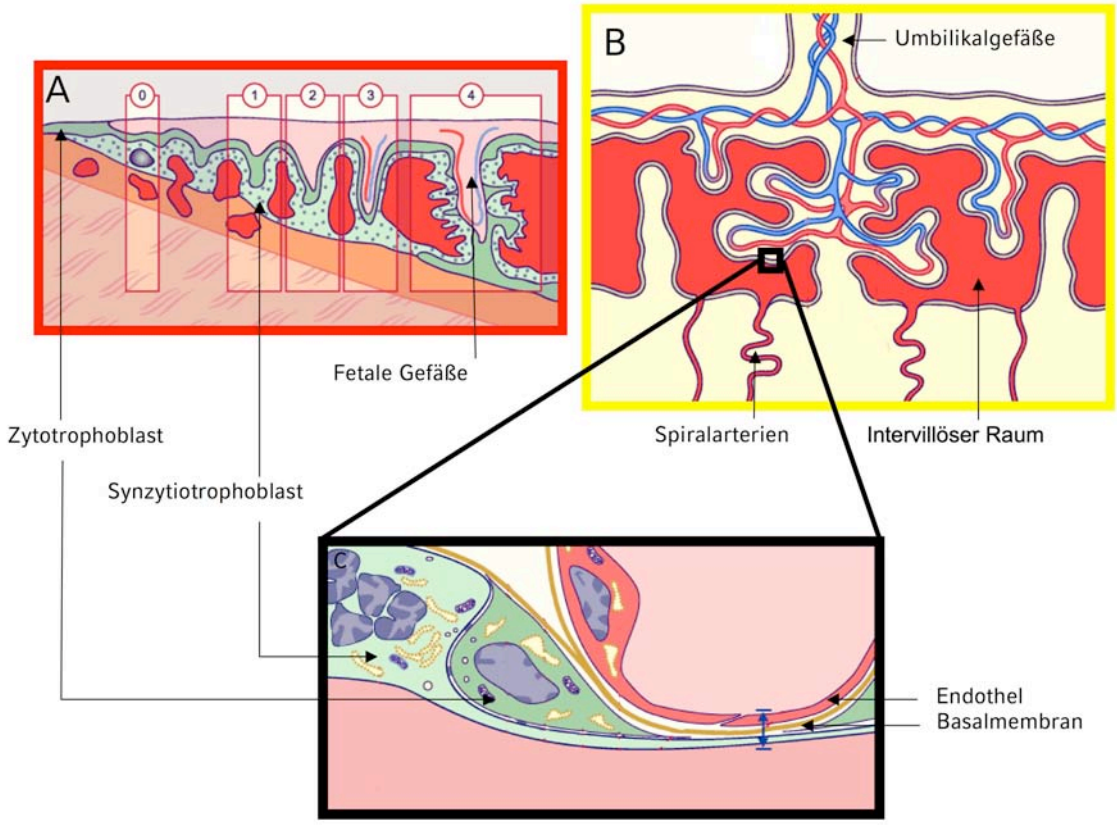


Ab dem 10. Entwicklungstag hat die invadierende Blastozyste mit der umgebenden Trophoblastenschale die mütterlichen Blutgefäße erreicht. Es beginnt der Aufbau der fetomaternalen Grenzzone. Adhäsion, Invasion und Wachstum werden im Wechselspiel zwischen Trophoblast und mütterlichen Zellen gesteuert. (Abbildung adaptiert nach [16])

Die humane Plazenta ist durch eine extrem tiefe Infiltration des mütterlichen Stromas (Dezidua) durch den Trophoblasten gekennzeichnet. Kein anderes höheres Säugetier (Eutheriae) besitzt eine solche enge Verzahnung von kindlichem und mütterlichen Gewebe[15]. Tief in der mütterlichen Dezidua finden sich trophoblastäre Zellnester, die sog. extravillösen Trophoblasten (EVT). Trophoblastzellen sind sekretorisch hochaktiv und bilden neben dem bekannten „Schwangerschaftshormon" βHCG eine Vielzahl weiterer Signalproteine darunter auch immunaktive Moleküle wie z.B. Zytokine und Chemokine und formen damit ihre immunologische Umgebung (Abbildung 3).

Ziel dieses invasiven Prozesses ist es, Anschluss an den mütterlichen Blutkreislauf zu gewinnen und einen geregelten Stoffaustausch über die Plazentaschranke aufzubauen (Abbildung 2). Eine angepasste Regulation des Blutflusses an der fetomaternalen Grenzfläche ist eine der wichtigsten Determinanten einer über 40 Wochen entwicklungsfähigen Plazenta. In diesem Invasions- und Arrosionsprozess wird der Trophoblast unterstützt vom spezifischen immunologischen Milieu, das die immunkompetenten Zellen der fetomaternalen Grenzzone aufbauen[16].

Abb 2. Plazentaentwicklung und Aufbau der fetomaternalen Austauschzone



A: Histologische Entwicklung der Plazenta von links nach rechts. Die verschiedenen Stadien (0-4) entwickeln sich nacheinander und treten nie zum gleichen Zeitpunkt auf. Am Ende der Entwicklung steht die reife Plazenta.

B: Schematische Darstellung des Baus einer ausgereiften Haftzotte am Ende der Schwangerschaft.

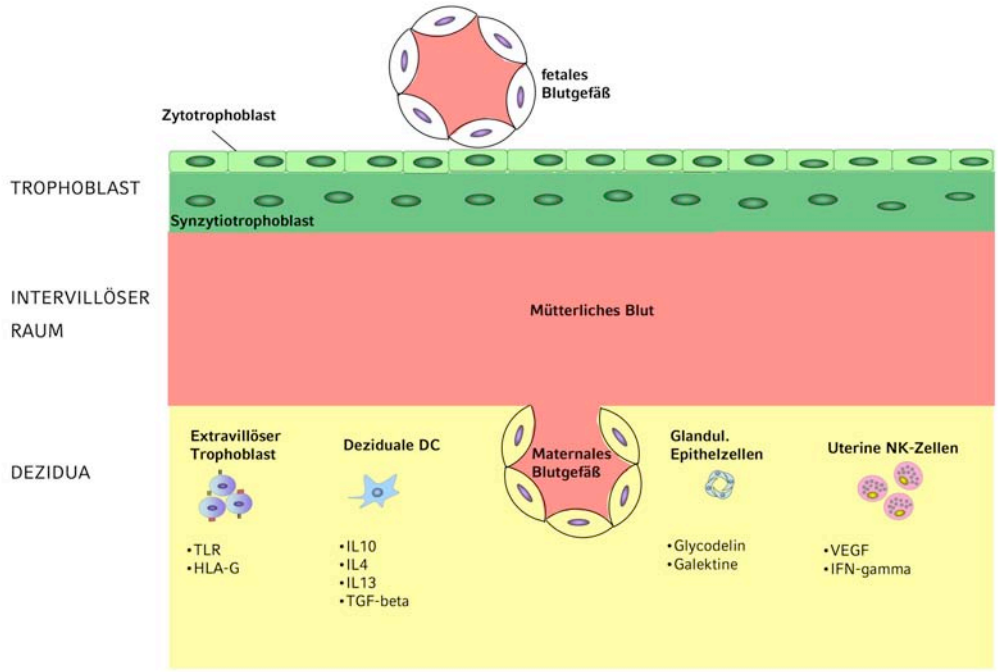
C: Mikroskopische Struktur der Plazentaschranke am Ende der Schwangerschaft. Die Plazentarschranke reduziert sich an der dünnsten Stelle (Blauer Pfeil) auf den kernlosen Synzytiotrophoblasten, die vereinigte Basalmembran und das Endothel. Es kommt zu einer direkten Interaktion zwischen kindlichem Trophoblasten und mütterlichen Blutzellen (Abbildungen adaptiert nach [5])

Immunanatomie der fetomaternalen Grenzzone

In der Dezidua selbst findet sich eine dichte Population immunkompetenter Zellen. Zudem spielt das plazenta-spezifisches Zyto- und Chemokinnetzwerk, welches durch diese Zellen konstituiert wird, eine zentrale Rolle (Abbildung 3).
Eine eigentliche organspezifische Infektabwehr lässt sich nur unter Rekrutierung externer Effektorzellen bewerkstelligen und stört massiv das plazentare Immunmilieu. Der gravide Uterus ist damit – ebenso wie z.B. die vordere Augenkammer - eine immun-privilegierte Zone, in der die Balance zwischen „Organerhalt" und „Infektabwehr" extrem in Richtung „Organerhalt" verschoben ist. In der vorderen Augenkammer gelingt z.B. ein allogenes Korneatransplantat unter sterilen Bedingungen, ohne dass es zu einer Abstoßungsreaktion kommt, während immunologische Gefahrensignale z.B. durch eine manifeste Infektion zum Immunzellinflux und oft zum permanenten Funktionsverlust der Kornea d.h. zur Erblindung führen[25].

Abb 3. Immun-Architektur der fetomaternalen Austauschzone

Der Trophoblast kommt in direkten Kontakt mit mütterlichem Blut im intervillösen Raum. In der Dezidua findet sich ein spezielles zelluläres immunologisches Milieu. Dargestellt ist eine Auswahl der zellulären Komponenten und ihre Hauptsignalmoleküle. (Darstellung angelehnt an[15])



Uterine Natürliche Killerzellen

$CD56^+$ Zellen sog. natürlichen Killerzellen (NK-Zellen) stellen eine Hauptkomponente des angeborenen Immunsystems dar. Sie zerstören -ohne Erkennen eines spezifischen Antigens- jene somatischen Zellen, deren Antigenpräsentations-Moleküle (HLA-Moleküle) genetisch „fremd" kodiert, infektionsbedingt funktionsgestört oder durch anhaftende Antikörper markiert sind. Die schnelle und nicht-antigenabhängige Vernichtung solcher Zellen stellt einen wichtigen Schutz gegen Viruserkrankungen dar, birgt aber ebenfalls die Gefahr der Auto-Immunität. Die zytotoxischen Mechanismen der NK-Zellen sind daher sehr strikt reguliert und ihre Ausreifung erfordert ein spezifisches, z.B. durch Zytokine und Chemokine geformtes immunologisches Milieu. Neben ihren zytotoxischen Fähigkeiten sind sie jedoch auch selbst wichtige Produzenten immunologischer Botenstoffe.

Uterine NK-Zellen (uNK Zellen), die etwa 70% der Immunzellen an der fetomaternalen Grenzzone ausmachen, stellen eine, durch das deziduale immunologische Milieu geprägte, Sonderform dar, die nur sehr wenig mit NK-Zellen des peripheren Blutes gemeinsam haben[20].

uNK Zellen sind deutlich weniger zytotoxisch und deutlich mehr sekretorisch aktiv. Ein Grund dieser unterschiedlichen Entwicklung von $CD56^+$ Zellen an der Dezidua ist auch jene spezielle trophoblastäre Form von antigenpräsentierenden Molekülen (HLA-Molekülen), die die zytotoxische Aktivität der NK-Zellen hemmen. Hier ist v.a. HLA-G zu nennen[15]. Lytisch tätig werden uNK Zellen v.a im Rahmen des Umbaus der Spiralarterien[17]. Die Bezeichnung „Killerzelle" und v.a. eine Gleichsetzung mit den ansonsten so bezeichneten Zellen des peripheren Blutes beschreibt die bislang bekannten physiologischen Eigenschaften der uNK-Zellen jedenfalls nur sehr unzureichend[10]. Die Sekretion von Interferon-γ durch $CD56^+$ Zellen, scheint beim Aufbau der plazentaren Immun-Architektur eine entscheidende Rolle zu spielen. Daneben sezernieren diese Zellen auch größere Mengen anderer vasoaktiver Substanzen wie z.B. *vascular endothelial growth factor* (VEGF), die ihrerseits bei der Vaskularisation der Plazenta eine entscheidende Rolle spielen[18].

Glanduläre Epithelzellen

An der Immun-Architektur der Plazenta entscheidend beteiligt sind ebenfalls glanduläre Epithelzellen[11]. Sie sezernieren in z.T. sehr großen Mengen lokal immunaktive Substanzen wie z.B. Galektine und Glycodelin. Beide Moleküle sind Teil der glykosilierungsabhängigen Immunzell-Interakation[19]. Galektine sind eine Gruppe evulotorisch hochkonservierter, kohlehydrat-bindender

Proteine, die spezifische Kohlehydratmuster in angepasste Immunantworten übersetzen helfen. Es konnte beispielsweise gezeigt werden, dass Galektin 3, wenn es mit N-Glycan komplexiert, die Aktivierbarkeit von T-Zellen inhibiert. Dies erfolgt durch eine Reduktion der Rezeptordichte auf der Oberfläche von T-Zellen[7].

Glycodelin

Glycodelin (auch *placental protein 14*; PP14) ist ein sezerniertes Glykoprotein, das zur Großfamilie der Lipocaline gehört. In unterschiedlicher Glykosilierung kommt es vor allem in den glandulären Epithelzellen des Endometriums und der Dezidua, aber auch im Seminalplasma des männlichen Ejakulates sowie in Ovarien, Eileitern und der weiblichen Brust vor[21]. Die Sekretion der Gycodeline ist im weiblichen Körper strikt abhängig vom monatlichen Anstieg der Progesteronkonzentration in der zweiten Zyklushälfte bzw. in der Frühschwangerschaft. In Uterusspülungen während des weiblichen Zyklus ist Glycodelin lediglich ab der mittleren Sekretionsphase im sog. „Implantationsfenster" nachweisbar. Dann jedoch in einer hohen Konzentration von bis zu 12 mg/l. Im Falle einer Schwangerschaft steigert sich die Glycodelin-Konzentration in der Amnionflüssigkeit – durch Sekretion aus dezidualen Drüsenzellen – bis zu einem Wert von 125 mg/l in der 12. Schwangerschaftswoche. Desweiteren exprimieren maligne gynäkologische Tumore Glycodelin in unterschiedlicher Konzentration. Ein Beispiel hierfür ist, neben dem Endometrium-Karzinom, das Ovarial-Karzinom, in dem Glycodelin mit gleicher Proteinstruktur aber unterschiedlicher Glykosilierung sezerniert wird. Glycodelin hat eine deutliche immunmodulatorisch Potenz. Glycodelin hat daher weitreichende Auswirkungen auf das lokale immunologische Milieu. Dies gilt sowohl für die zelluläre Zusammensetzung als auch für das Chemo- und Zytokinprofil[14, 22].

Zytokine und Chemokine

Zytokine nehmen Einfluss auf die Implantation sowie die fetale Entwicklung und Differenzierung. Zu Beginn der Zytokinforschung fokussierte man sich auf die Zytokinsekretion durch T-Zellen. Es konnte nachgewiesen werden, dass $CD4^+$ T-Helfer Zellen abhängig von der Art der Infektion unterschiedliche Zytokine produzieren können und nannte zwei experimentell gut zu unterscheidenden Pole der Immunantwort „Th1" und „Th2". Th1-Zellen sezernieren Zytokine wie Interleukin (IL-)2, Tumornekrose Faktor (TNF)-beta und Interferon-γ. Th2-Zellen sezernieren IL-4, IL-5 und IL-10. Beide, Th1- und Th2-Zellen können u.a.

IL-3, -6, -12, -13, TNF-alpha und GM-CSF freisetzen. Th1-Zytokine begleiten zytotoxische Immunreaktionen. Klassischerweise als Antwort auf eine virale Infektion werden dabei infizierte Zellen durch zytotoxische T-Zellen zerstört. Th2-Zytokine verstärken auf der anderen Seite die Antikörperproduktion (insbesondere IgE) sowie die Proliferation und Funktion der Eosinophilen, was die adäquate Immunreaktion z.B. auf einen Parasitenbefall darstellt. Th2-Antworten unterdrücken Th1-Antworten und umgekehrt. Wegmann und Mitarbeiter entwickelten die Theorie einer Balance zwischen Th1/Th2-Zytokinen und betonten, dass das fetale Überleben von einer Inhibierung der Th1-Antwort durch Th2-Zytokine abhängt[28]. In den folgenden Jahren haben sich viele Forschungsarbeiten darauf fokussiert, den Einfluss von (infekt-induzierten) Th1-Zytokinen auf das Abortgeschehen darzulegen. Obwohl diese Studien gezeigt haben, dass Zytokine einzigartige und wichtige Effekte in der Schwangerschaft ausüben, hat sich das Th1/Th2 Paradigma gewandelt. Zytokinnetzwerke sind in hohem Maße synergistisch und redundant aufgebaut und die Untersuchung einzelner Zytokine liefert oftmals keine eindeutigen Ergebnisse. Die ehemals dogmatische Theorie einer Veränderung der Th1/Th2 Balance in der normalen und gestörten Schwangerschaft ist dadurch deutlich vielschichtiger geworden. Th1-vermittelte Autoimmunerkrankungen (z.B. die rheumatoide Arthritis) bessern sich oftmals in der Schwangerschaft, während Th2 vermittelte (z.B. der systemische Lupus erythematodes) sich in der Schwangerschaft oftmals verschlechtern. Neuere Untersuchungen sehen hierin jedoch eher ein Epiphänomen eines veränderten Hormon- und Zytokinhaushalts, als einen Beweis dafür, dass ein systemisches Th2-Zytokinmuster essentiell für das erfolgreiche Austragen einer Schwangerschaft ist.

Trophoblast

Der Trophoblast exprimiert Pathogen-Erkennungs-Rezeptoren (u.a. sog. Toll-Like Rezeptoren). Diese phylogenetisch sehr alten Rezeptoren - sie finden sich schon im Immunsystem der Fruchtfliege - erkennen charakteristische Bestandteile von Erregern und veranlassen trophoblastäre Zellen, eine gewebs- und erreger-spezifische Immunantwort auszulösen[12]. Diese Rezeptoren sind bildlich gesprochen die „Schalter", die bei einer manifesten Infektion die Infektabwehr in Gang setzen. Sie werden v.a. in den Zellnestern des extravillösen Trophoblasten exprimiert, so dass lediglich Infektionen tieferer Schichten der Dezidua eine Immunantwort nach sich ziehen[1]. Im Ruhezustand sezernieren die trophoblastären Zellen jedoch Zytokine und lokal aktive Chemokine und tragen auf ihrer Oberfläche ein spezifisches Muster

antigenpräsentierender Moleküle (z.B. HLA-G). Dieses prägt klassische Immunzellen so, dass sie die Implantation und Invasion unterstützen und steuern.

Antigenpräsentierende Zellen

Dendritische Zellen (DC) besetzen eine zentrale Schaltstelle des Immunsystems und können einerseits antigenspezifische zytotoxische T-Zell Immunantworten induzieren und andererseits im *steady state* für immunologische Toleranz sorgen[3, 24]. Es ist daher nicht verwunderlich, dass DC auch in der Dezidua eine große Zellpopulation darstellen. DC nehmen konstant Fragmente apoptotisch zugrunde gegangener Zellen auf und präsentieren deren Antigene, MHC-restringiert, auf ihrer Oberfläche. Sie präsentieren diese (auch fetalen) Antigene in einem immunologischen Kontext, der antigen-spezifische Immunantworten, z.B. durch die Generierung regulatorischer T-Zellen, unterdrückt. Bei einer Infektion kommen jedoch DC in Kontakt mit Gefahrensignalen, die eine radikale Zustandsänderung bewirken. Aktivierte DC wandern in regionäre Lymphknoten und kommen dort in Kontakt mit Effektorzellen des adaptiven Immunsystems. Sie präsentieren das aufgenommene Antigen in einer Weise, die diese Effektorzellen (z.B. zytotoxische T-Zellen) zur klonalen Expansion anregt und welche ihrerseits im infizierten Organ die betroffenen Zellen zielgerichtet zerstören können. Im „*steady state*" tragen deziduale DC wesentlich zur physiologischen Immun-Architektur der Plazenta bei[4].

Eigene Ergebnisse

Dendritische Zellen

Physiologie

Schnurr M, Then F, Galambos P, ***Scholz C****, Siegmund B, Endres S, Eigler A.*
Extracellular ATP and TNF-alpha synergize in the activation and maturation of human dendritic cells.
J Immunol. 2000; 165:4704-4709.

Ob dendritische Zellen einen immunogenen oder tolerogenen Phänotyp annehmen, entscheidet sich je nach dem, welches Gefahrensignal sie registrieren. Die Charakteristik des Gefahrensignals bedingt neben dem spezifischen Antigen dann eine angepasste Immunantwort. In diesem Zusammenhang konnten wir die DC-aktivierende Potenz der Pyrmidinderivate ATP und ADP näher untersuchen. Es war seit längerem bekannt, dass entsprechende Rezeptoren auf DC vorhanden waren. Die genaue Reaktion von DC nach Ligation dieser Rezeptoren war jedoch unklar. Wir konnten zeigen, dass extrazelluläres ATP einen synergistischen Effekt auf die Stimulation von DC hat. ATP ist ein strikt intrazelluläres Molekül und ein Auftauchen im extrazellulären Raum wird von DC registriert und mit einer Immunreaktion beantwortet. ATP erhöhte dabei transient die endozytotische Aktivität sowie die IL-12 Sekretion und T-Zell stimulierende Kapazität von DC. Dieser Effekt war in Kombination mit TNF-alpha stärker ausgeprägt. Zusätzlich konnte durch Blockung mit einem spezifischen Pyrimidinrezeptor-Antagonisten eine Rezeptorspezifität nachweisen werden.

Reproduktionsimmunologie

Scholz C, *Toth B, Brunnhuber R, Rampf E, Weissenbacher T, Santoso L, Friese K, Jeschke U.*
Glycodelin A induces a tolerognic phenotype in monocyte-derived dendritic cells in vitro.
Am J Reprod Immunol. 2008 Dec;60(6):501-12.

Im Gegensatz zu ATP verändern DC ihren Phänotyp bei einer Interaktion mit schwangerschafts-assoziiertem Glycodelin (Gd) in gänzlich anderer Weise. Dieses Protein musste zunächst aus Amnionflüssigkeit mittels *High-performance liquid chromatography* (HPLC) gereinigt werden, da die spezifische Zuckerstruktur dieses Glykoproteins zu seiner Wirkung entscheidend beiträgt und es daher nicht rekombinant hergestellt werden kann. In nachfolgenden Experimenten mit humanen monozytenabgeleiteten DC zeigte sich ein charakteristisches Muster reifungsassoziierter Moleküle. CD83 und CD86 - zwei Moleküle, die eine Ausreifung anzeigen - waren bei der Inkubation mit Gd nicht erhöht, obwohl nachfolgend mit TNF-alpha versucht wurde eine Reifung herbeizuführen. Insgesamt zeigte sich bei diesen Experimenten ein Phänotyp, der am ehesten eine tolerogne Potenz vermuten ließ[24]. In der Tat waren funktionelle Assays, nämlich die T-Zell stimulierende Potenz sowie die Zytotoxizität DC-stimulierter Lymphozyten immer dann abgeschwächt, wenn DC mit Gd präinkubiert waren. Gd war dabei im experimentellen Ansatz lediglich am Beginn des Reifungsprozesses vorhanden. Der tolerogene Effekt auf DC war jedoch auch ohne aktuelle Gd-Exposition stabil. Ein direkter Nachweis einer Rezeptorvermittlung dieses Effekts gelang uns allerdings experimentell nicht. Interessanterweise war in unseren Experimenten der *C-type Lectin* Rezeptor DC-SIGN in jenen Ansätzen signifikant erhöht, in denen DC mit Gd präinkubiert waren. Arbeiten, die eine Analyse der spezifischen Affinität dieses Rezeptors durchgeführt hatten, waren jedoch zu dem Ergebnis gekommen, dass die Zuckerstruktur eines fucosylierten Lac-di-NAc-N-Glycans die höchste Bindungsaffinität aufwies[26]. Diese Zuckerstruktur exisitert beim Menschen - soweit bislang bekannt - ausschließlich auf Gd. Ansonsten findet sich diese spezifische Verzuckerung beim Helminten insbesondere beim Pärchenegel *Schistosoma mansoni*[27]. Die hohe IL-10 Sekretion – ein Zytokin, das typischerweise bei Wurmerkrankungen erhöht ist - durch DC nach Inkubation mit Gd legt eine Verbindung zwischen dem immunolgischen Milieu der Plazenta und der Immunantwort einer parasitären Erkrankung mit Schistosomen nahe.

Toth B, Roth K, Kunert-Keil C, ***Scholz C****, Schulze S, Mylonas I, Friese K, Jeschke U.*
Glycodelin protein and mRNA is downregulated in human first trimester abortion and partially upregulated in mole pregnancy.
J Histochem Cytochem. 2008 May;56(5):477-85.

Wenn Gd, das immunogne Verhalten von DC so deutlich ändert und auch auf anderen Zellen des dezidualen Immunmilieus – insbesondere bei uNK Zellen – eine Wirkung entfaltet, so stellt sich die Frage, ob bei einer gestörter Plazentation eine Veränderung der Gd-Konzentration zu verzeichnen ist. Daher wurde Gewebe aus der normalen und gestörten Früh-Schwangerschaft (Abort und Molenschwagerschaft auf die Expression von Gd untersucht. Im Fall eines Abortes konnte eine erniedrigte Konzentration von Gd im Fall einer Molen-Schwangerschaft eine erhöhte Konzentration nachgewiesen werden. Anhand von *in vitro* Experimenten konnten wir zusätzlich eine Vermittlung über den Gonadotropinrezeptor und eine Abhängigkeit von der βHCG-Konzentration nachweisen.

Scholz C*, Toth B, Santoso L, Kuhn C, Franz M, Mayr D, Jeschke U, Friese K, Schiessl B.*
Distribution and maturity of dendritic cells in diseases of insufficient placentation.
Am J Rep Immunol 2008; 60:238–245.

Frühaborte, also das Absterben der Schwangerschaft im ersten Trimenon, sind (abhängig vom maternalen Alter) in der überwiegenden Mehrzahl der Fälle genetisch bedingt. So kann eine Veränderung des Plazentamilieus immer auch als Epiphänomen gesehen werden. Im Gegensatz dazu sind die hypertensiven Schwangerschaftserkrankungen, die in der Spätphase der Schwangerschaft auftreten, Ausdruck einer bereits in der Frühschwangerschaft fehlgesteuerten Plazentation. Dabei unterbleibt als zentraler pathophysiolgischer Mechanismus der Umbau der Spiralarterien von einem Hochdrucksystem hin zu einem Niederdrucksystem mit venösen Charakteristika und einem Mitteldruck im intervillösen Raum am Ende der Schwangerschaft von ~10 mmHg. Dieser Umbau wird erzielt durch das Ersetzen der *Muscularis* durch Trophoblastzellen, bei dem uNK Zellen eine zentrale Rolle spielen. Dieser Ersatz kann nur im physiologischen immunologischen Milieu stattfinden, deren Teil das Trophoblastensystem ist. So verwundert es nicht, dass der Reifegrad von DC und die Verteilung innerhalb der Dezidua im Rahmen von hypertensiven Schwangerschaftserkrankungen verändert sind. Im Rahmen hypertensiver Schwangerschaftserkrankungen gelang es uns als erste Arbeitsgruppe, eine

Rolle des Aktivierungszustandes dendritischer Zellen am Patientenmaterial zu zeigen. DC zeigen dabei einerseits eine höhere Dichte jenes Rezeptors DC-SIGN, wie wir es bereits bei *in vitro* Experimenten mit Gd gesehen hatten. Auf der anderen Seite konnten nachweisen werden, dass DC bei hypertensiven Schwangerschaftserkrankungen mit uNK Zellen co-lokalisieren und zusätzlich *VEGF-R* 1 und 2 überexpimieren. Wir konnten damit zum ersten Mal eine Verbindung zwischen plazentaren DC und den (fehlgesteuerten) Vaskularisationsprozessen der Plazenta zeigen.

Tumorimmunologie

Schnurr M, Galambos P, ***Scholz C****, Then F, Dauer M, Endres S, Eigler A.*
Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines.
Cancer Res. 2001; 61:6445-6450.

DC als zentrale Steuereinheiten des adaptiven Immunsystems waren seit ihrer Entdeckung durch R. Steinman 1973 als Träger einer tumorgerichteten Immunantwort in der Diskussion[23]. Viele Studien wurden zwischenzeitlich an Patienten durchgeführt, ohne dass bislang eine routinemäßige klinische Anwendung außerhalb klinischer Studien erkennbar wäre. Auf der Suche nach *in vitro* Testsystemen konnten wir für die Anwendung mit Lysat von Tumorzellen ein diesbezügliches System etablieren. Über eine Dauer von 6 Wochen wurden autologe T-Zellen mit antigenbeladenen DC so stimuliert, dass sich mit ihnen eine Anti-Tumor-Immunantwort *in vitro* generieren ließ. Mit diesem Testsystem war es nun möglich, Determinanten einer DC-gestützten Immuntherapie, z.B. Fragen des optimalen Reifestimulus oder der Antigenbeladung systematisch anzugehen.

Schnurr M, ***Scholz C****, Rothenfusser S, Galambos P, Dauer M, Robe J, Endres S, Eigler A.*
Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells.
Cancer Res. 2002; 62:2347-2352.

Die Art und Weise, wie eine DC Antigen aufnimmt, determiniert die daraus folgende Immunantwort. Für eine tumorgerichtete Immunantwort ist dabei eine von zytotoxischen T-Zellen (CTL) getragene Immunantwort sehr vorteilhaft. DC sind die einzigen Zellen des Immunsystems, die eine solche CTL-Immunantwort *de novo* generieren können. Ihre Eigenschaft der spezifischen Immuninduktion

prädestinieren DC für eine individualisierte Krebs-Immuntherapie. Deren immunogene Eigenschaften konnten wir in Zellkultur-Modellen beurteilen. Apoptose, als der physiologische Zelluntergang, induziert peripher (d.h. außerhalb lymphatischer Organe), vermittelt über DC, immunologische Toleranz. Apoptotisch zu Grunde gegangene Zellen werden dabei von DC aufgenommen und so aufbereitet, dass ihre charakteristische Proteinstruktur von CTL erkannt wird. Zusätzliche Signale bestimmen nun, ob diesen T-Zellen angezeigt wird, die betreffende Proteinstruktur zu tolerieren oder dagegen eine Immunantwort zu induzieren. Eine solche Immunantwort ist hochspezifisch und bietet sich daher als *„targeted therapy"* in der Krebstherapie an. Wir konnten in diesem Zusammenhang den Weg apoptotischen Tumormaterials in Zellkultur-DC genauer verfolgen und als Einflussfaktor der folgenden Immunantwort näher charakterisieren.

Die Generierung einer solchen CTL-Immunantwort durch phagozytierte Antigene wird als *cross-priming* bezeichnet. Wir konnten zeigen, dass für apoptotische Antigene ein solches *cross-priming* stattfindet, wohingegen dies für nekrotisch zugrunde gegangenes Material nicht der Fall ist.

***Scholz C**, Toth B, Barthell E, Mylonas I, Weissenbacher T, Friese K, Jeschke U.*
Immunohistochemical expression of glycodelin in breast cancer correlates with estrogen-receptor alpha and progesterone-receptor A positivity.
Histol Histopathol. 2009 Apr;24(4):467-71.

*Jeschke U, Mylonas I, Kunert-Keil C, Stahn R, **Scholz C**, Janni W, Kuhn C, Schröder E, Mayr D, Friese K.*
Immunohistochemistry, glycosylation and immunosuppression of glycodelin in human ovarian cancer.
Histochem Cell Biol. 2009 Feb;131(2):283-95.

***Scholz C**, Rampf E, Toth B, Brunnhuber R, Weissenbacher T, Friese K, Jeschke U*
Ovarian Cancer-derived Glycodelin Impairs In Vitro Dendritic Cell Maturation
J Immunother. 2009 Jun;32(5):492-7.

In einer weiteren Serie von Experimenten versuchten wir, den Einfluss des bereits an der fetomaternalen Grenzzone charakterisierten Gd auf die Pathophysiologie maligner gynäkologischer Erkrankungen zu beschrieben. Interessanterweise spielt Gd sowohl beim Mamma-Karzinom aber v.a. auch beim Ovarial-Karzinom eine Rolle. Beim Brustkrebs scheint dies insbesondere bei jenen histologischen Typen der Fall zu sein, bei denen noch ein gewisser Grad an Differenzierung vorhanden ist. Es kommt zu einer Korrelation zwischen

der Expression von Hormonrezeptoren und der vermehrten Sekretion von Gd. Erste funktionelle Daten konnten wir mit aus malignem Aszites gewonnenem Gd generieren. Zunächst wiesen wir eine differenzierte Glykosilierung von Ovarial-Karzinom-Aszites-Gd vs. Amnionflüssigkeit-Gd nach. Die Glykosilierung hat ihrerseits einen entscheidenden Einfluss auf das Färbeverhalten in der Immunhistochemie aber v.a. auch auf die Rezeptorbindung z.B. an Muster-Erkennungs-Rezeporen wie DC-SIGN. Auch das different glykosilierte Ovarialkarzinom-Gd zeigte einen deutlichen Effekt auf DC. Auch hier zeigt sich ein tolerogener Phänotyp, der hier nun – so unsere Interpretation - den maligne entarteten Zellen hilft ihr immunogenes Milieu zu formen. Hier scheint tatsächlich eine Nutzung im Rahmen der Plazentation erprobter Mechanismen im Kontext malignen Wachstums stattzufinden.

Krankheitsbilder

Scholz C, *Hermann C, Toth B, Kachler A, Kainer F, Friese K, Makrigiannakis A, Jeschke U*
Association of placental inflammation with fetomaternal hemorrhage and loss of placental mucin-1
Eingereicht bei Gynecologic and Obstetric Investigation

Scholz C, *Kachler A, Hermann C, Weissenbacher T, Toth B, Friese K, Kainer F.*
Flowcytometric assessment of fetomaternal hemorrhage during external cephalic version at term.
J Perinat Med. 2009;37(4):334-7.

Eine der zentralen Aufgaben der fetomaternalen Grenzfläche ist die Trennung des mütterlichen und kindlichen Blutkreislaufes. Bei einem Leck dieser Trennung kann es zum Ausbluten des Feten in den Kreislauf der Mutter kommen. Mechanische Belastung wurde lange Zeit als ein Hauptriskofaktor für dieses seltene, jedoch in seinem Verlauf oftmals sehr dramatische Krankheitsbild gesehen. In einer Beobachtungsstudie konnten wir mit einem sehr sensitiven durchflußzytometrischen Testverfahren jedoch eine plazentare Entzündungsreaktion als bislang nicht beschriebenen Risikofaktor etablieren.

Ein lange Zeit mit besonderer Aufmerksamkeit verfolgter Risikofaktor der fetomaternalen Transfusion war die mechanische Belastung im Rahmen der sog. äußeren Wendung, bei der ein Kind am Ende der Schwangerschaft aus Beckenendlage durch Manipulation von außen in eine Schädellage gedreht wird, um eine vaginale Geburt aus Schädellage zu ermöglichen. Die Sicherheit des Kindes steht dabei naturgemäß an oberster Stelle. Ebenfalls in einer klinischen Beobachtungs-Studie konnten wir mit o.g. Testverfahren dazu beitragen die Volumina der fetomaternalen Transfusion im Rahmen einer äußeren Wendung mit genauer zu quantifizieren und den Einfluss der mechanischen Belastung auf die fetomaternale Transfusion damit zu relativieren

Ausblick

Derzeitig laufende Projekte beschäftigen sich mit der lokalen Immunreaktion des epithelialen Ovarialkarzinoms. Neueste noch unveröffentlichte Daten zeigen, dass in einer multivariaten Analyse die Sekretion von Gd bei Patientinnen mit einer epithelialen ovariellen Neoplasie pT3c signifikant mit dem Gesamtüberleben korreliert. Ebenso untersucht wird die Regulation des Chemokins CCL22, von dem durch Curiel et al. ebenfalls gezeigt werden konnte, dass seine Expression mit dem Gesamtüberleben von Ovarialkarzinompatientinnen korreliert[6]. Die zuletzt genannten Experimente werden in Fortführung der Kooperation der Frauenklinik mit der Abteilung für Klinische Pharmakologie der LMU durchgeführt.

L Santoso, C Kuhn, S Kunze, K Friese, U Jeschke, C Scholz
TLR2-ligation Listeria moncytogenes and its effects on the cytokine profile of trophoblast cells
Manuskript in Vorbereitung

Reproduktionsimmunologisch beschäftigen wir uns derzeit mit der trophoblastären Reaktion auf Liganden für Mustererkennungsrezeptoren. Gerade abgeschlossene Experimente zeigen eine differenzierte Expression und Reaktion des ST und ZT auf eine TLR-2 Ligation mit *Listeria moncytogenes*. Interessanterweise führt eine Ligation von TLR-2 beim Trophoblast zu keiner angepassten Immunantwort, sonder fördert eher die Vermehrung von *L. moncytogenes*. Dies könnte dazu beitragen, das Phänomen des Plazentatropismus von *L. monocytogenes* zu erklären. Während diese Erkrankung außerhalb der Schwangerschaft in der Regel sehr harmlos verläuft, kommt es in der Schwangerschaft zu einer selektiven Vermehrung der Listerien in der Plazenta[2]. Erst dieser immunologisch sichere Hafen macht die Erkrankung in der Schwangerschaft zu einer Gefahr für die Mutter aber insbesondere auch für das Kind.

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Abkürzungsverzeichnis

ATP	Adenosin-5´triphosphat
ADP	Adenosin-5´diphosphat
CD	*Cluster of differentiation*
$CD(x)^+$	Positiv für einen Oberflächenmolekül x
CTL	zytotoxische T Zelle
DC	Dendritische Zelle
ELISA	*Enzyme-linked immunosorbent assay*
FACS	*Fluorescence-activated cell sorter*
Gd	Glycodelin
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
HLA	Humanes Leukozyten-Antigen
IU	Internationale Einheit
IL	Interleukin
MHC	*Major histocompatibility complex*
min	Minute
O_2	Sauerstoff
ST	Syzytiotrophoblast
TLR	Toll-Like Rezeptor
TNF-alpha	Tumornekrosefaktor-α
uNK Zelle	uterine natürliche Killerzelle
VEGF	*vacular endothelial growth factor*
ZT	Zytotrophoblast

Anhang

Lebenslauf

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Apoptotic Pancreatic Tumor Cells Are Superior to Cell Lysates in Promoting Cross-Priming of Cytotoxic T Cells and Activate NK and γδ T Cells[1]

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ABSTRACT

Tumor vaccines using dendritic cells (DCs) have been shown to induce antitumor CTL responses. The choice of the tumor antigen preparation used for DC loading is still an unresolved issue. We compared DCs pulsed with cell lysates, whole apoptotic tumor cells or their supernatants of the HLA-A2$^+$ human pancreatic carcinoma cell line Panc-1 for their capacity to activate T cells. Monocyte-derived DCs from HLA-A2$^+$ donors were pulsed with tumor antigen, matured subsequently, and cocultured with autologeous peripheral blood mononuclear cells. After three weekly restimulations with DCs, T-cell activation was assessed by intracellular IFN-γ staining and cytotoxicity assays. Compared with lysate, pulsing DCs with the supernatant of apoptotic tumor cells induced a higher frequency of activated CTLs and T-helper cells, as well as an enhanced MHC class I-restricted tumor cell lysis. No activation of natural killer (NK) or γδ T cells was detected. Pulsing DCs with whole apoptotic tumor cells induced an even more pronounced lytic effect. However, in this case, MHC class-I blocking was only partially effective, and unrelated cell lines were also killed. IFN-γ staining revealed activation of CTLs and T-helper cells, as well as NK and γδ T cells. *Trans*-well cultures of NK cells, apoptotic tumor cells, and DCs showed that NK cell activation was dependent on direct cell-to-cell contact with tumor cells and the presence of interleukin-12 produced by DCs. These results indicate that the choice of antigen preparation is a critical determinant in the induction of antitumor immunity. Tumor vaccines consisting of DCs and apoptotic tumor cells may be able to activate CTLs, as well as effector cells of the innate immune system.

INTRODUCTION

Tumor vaccines aim at inducing CTL responses against tumor antigens to mount an immune response against tumors. The activation of CTL requires presentation of the antigen in the context of MHC molecules on the surface of antigen-presenting cells. DCs[4] are the most effective antigen-presenting cells (1), and tumor vaccinations with DCs have been shown to induce CTL responses and tumor regression in some patients (2–6). In contrast to CTL, NK cells and γδ T cells, effector cells of the innate immune system, have the capacity to recognize and kill tumor cells in an antigen-independent manner. There is evidence that DC can link innate and acquired effector mechanisms (1) either by direct cell-to-cell contacts (7) or by secreting cytokines (8, 9). Activation of effector cells of the innate immune system by DC might be a prerequisite for antitumor immunity in MHC class-I-negative tumors (7) and, under certain circumstances, for successful CTL responses (10).

Compared with tumors, such as renal cell carcinoma and malignant melanoma, pancreatic carcinoma is considered to be weakly immunogenic. In addition, pancreatic cancer cells exhibit several mechanisms of immune evasion, such as the expression of the apoptosis-inducing molecule Fas-ligand (11), defective signaling via Fas (12), and secretion of transforming growth factor-β, a growth factor known to interfere with DC function (13). Nevertheless, as we have shown previously, CTL responses against pancreatic cancer cells can be induced *in vitro* using DCs (14). Therefore, vaccination with DCs might offer a therapeutic option for patients with pancreatic carcinoma.

A critical issue in optimizing DC vaccines is the choice of tumor antigen for DC loading. Clinical vaccination trials for patients with malignant melanoma have demonstrated that vaccinating against a single antigen can induce tumor-specific CTLs but carries the risk of promoting tumor antigen escape variants (6). A more recent trial showed that the generation of CTLs against three or more tumor antigens correlates with clinical response (15). However, for many tumors, no or only few antigenic epitopes are known. To circumvent this limitation, whole tumor cells containing a spectrum of known, as well as yet unknown antigens, might be used as an antigen source. The additional presence of epitopes for T-helper lymphocytes could be beneficial because MHC II-restricted activation of T-helper lymphocytes plays a pivotal role in the physiological immune response to pathogens and might be of considerable importance in the process of tumor rejection (16).

Effective cross-priming with antigens from tumor cells has been demonstrated with tumor cell lysates (17), apoptotic tumor cells (18–22), and released particles from tumor cells, such as apoptotic bodies (23) and tumor-derived exosomes (24, 25). In the present study, we compared DCs pulsed with cell lysates, apoptotic cells, or released particles from apoptotic cells obtained from pancreatic carcinoma cells for their ability to cross-prime CTLs, as well as to activate T-helper and effector cells of the innate immune system.

MATERIALS AND METHODS

Media and Reagents. RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2% human serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (Life Technologies, Inc., Paisley, Scotland), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma Chemical Co., Munich, Germany) was used to culture cells from PBMCs. Recombinant human cytokines: granulocyte macrophage colony-stimulating factor was purchased from Novartis (Basel, Switzerland), IL-4 from Promega (Madison, WI), IL-2 and IL-7 from Strathman Biotech (Hanover, Germany), and TNF-α from R&D Systems (Wiesbaden, Germany). Prostaglandin E_2, CFSE, fluorochrome-labeled PKH-26, PI, and brefeldin A were obtained from Sigma Chemical Co. [^{3}H]thymidine was purchased from Amersham Buchler (Freiburg, Germany) and $Na_2[^{51}Cr]O_4$ from NEN Life Sciences (Zavantem, Belgium).

Isolation and Culture of Cells. PBMCs were obtained from peripheral blood or buffy coats of healthy HLA-A2$^+$ blood donors by Ficoll-Hypaque density gradient centrifugation. Monocyte-derived DCs were generated from the adherent fraction of PBMCs cultured in the presence of granulocyte macrophage colony-stimulating factor (1000 units/ml) and IL-4 (500 units/ml) for 6 days as described (14). NK cells were enriched from PBMCs by magnetically activated cell sorting using the isolation kit for untouched NK

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[4] The abbreviations used are: DC, dendritic cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor; IL, interleukin; PI, propidium iodide; CFSE, carboxy-fluorescein diacetate succinimidyl ester; mAb, monoclonal antibody; PGE_2, prostaglandin E_2; hsp, heat shock protein.

cells from Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of isolated $CD3^-$ $CD56^+$ NK cells was >95%. The human pancreatic carcinoma cell line Panc-1 (HLA-$A2^+$) and the NK cell-sensitive cell line K562 were purchased from European Collection of Animal Cell Cultures (Salisbury, Great Britain); the gastric carcinoma cell line Kato-III (HLA-$A2^+$) was obtained from American Type Culture Collection. Tumor cells were maintained in RPMI 1640 supplemented with 10% FCS (Biochrom), L-glutamine, penicillin, and streptomycin.

Preparation of Tumor Antigens. Tumor antigens were obtained from Panc-1 tumor cells. Tumor cell lysates were generated by three rapid freeze-thaw cycles as described (14). To induce tumor cell apoptosis, 3×10^6 tumor cells were suspended in medium and exposed to 150 mJ/cm^2 UV-B light or 43°C for 2 h. Subsequently, cells were cultured at 37°C on a shaker (30 rpm) to prevent cells from adhering to the culture dish. After 24–32 h, most tumor cells were early apoptotic, defined as annexin V positive and PI negative. By 48–72 h, all tumor cells had died staining positive for annexin V and PI. In our experiments, tumor cells were separated from their supernatant by centrifugation (10 min at 300 × *g*) after 24–32 h to obtain two fractions consisting of apoptotic tumor cells and low-density fragments, such as apoptotic bodies and other released tumor constituents.

Detection of Apoptosis. To detect apoptosis after heat or UV-B exposure, 2×10^6 tumor cells were stained with FITC-conjugated annexin V (Bender Med Systems, Vienna, Austria), and 1 μg/ml PI was added shortly before analysis by flow cytometry.

Tumor Antigen Uptake by DCs. Panc-1 tumor cells were stained with CFSE (1 μM) and DCs with PKH-26-FITC (2 μM) according to the manufacturer's protocols. Tumor cells were exposed subsequently to UV-B light or 43°C. After 24 h, equal numbers of tumor cells (or their supernatants) were incubated with DCs. Phagocytosis was assessed at various time points by flow cytometry and fluorescence microscopy in two-well chamber slides (Nunc, Wiesbaden, Germany). Intracellular uptake of tumor cell particles was confirmed by confocal laser microscopy (LSM 410 Invert; Carl Zeiss, Jena, Germany). Digital images were obtained in 10 focal planes separated by 1 μm and processed with PhotoShop 3.0 (Adobe Systems, San Jose, CA).

Coculture of DCs with Autologeous PBMCs. DCs were pulsed with tumor cell lysate, apoptotic tumor cells, or their supernatants (one tumor cell equivalent per DC) for 4 h, extensively washed, and incubated with TNF-α (1000 units/ml) and PGE_2 (1 μg/ml) for 24 h to induce a mature phenotype (26). These DCs were cocultured with autologeous nonadherent PBMCs at a ratio of 1:20. Every 7 days, the cultures were restimulated with new DCs. One-third of the medium was replaced on days 3, 5, and at each restimulation by fresh culture medium containing IL-2 (25 units/ml) and IL-7 (10 ng/ml).

Flow Cytometry. Surface antigen staining was performed as described previously (14). Fluorescence-labeled mAb against CD4, CD8, CD14, CD56, CD69, CD80, CD83, CD86, and HLA-DR was purchased from PharMingen (San Diego, CA), anti-CD3-PerCP from Becton Dickinson (San Jose, CA), and anti-Vγ9-FITC from Coulter Immunotech (Marseilles, France). For the detection of intracellular IFN-γ, cells were incubated with brefeldin A (1 μg/ml) during the last 4 h before harvest. Cells were stained with mAb against the surface markers of interest (CD3, CD4, CD8, or CD3, Vγ9, and CD56), fixed, permeabilized, and stained with anti-IFN-γ-phycoerythrin (Becton Dickinson).

Quantitation of Cytokine Secretion. Supernatants of the cocultures were harvested, and concentrations of human cytokines IL-12 (p40/p70; Bender Med Systems), IFN-γ, IL-10, and IL-4 were quantified by ELISA (Becton Dickinson) as duplicates.

Cytotoxicity Assay and MHC Class-I Blocking. After four weekly stimulations with DCs, the lytic activity of PBMCs was assessed in a ^{51}Cr-release assay. A suspension of single target cells was incubated with 100 μCi $Na_2[^{51}Cr]O_4/10^6$ cells for 1 h and washed five times. Tumor cells, 5×10^3/well, were incubated with PBMCs from the cocultures at E:T ratios ranging from 80:1 to 10:1 in round-bottomed, 96-well microtiter plates. Optimal incubation periods were determined for each target cell line by aiming for a low (experimental counts/spontaneous counts) ratio. The incubation period was 16 h for Panc-1 and Kato-III and 4 h for K562. Thereafter, 100 μl of supernatant of each well were collected, and radioactivity was measured with a gamma counter (Wallac Oy, Turku, Finland). Specific lysis was calculated by the formula: specific ^{51}Cr-release = [(experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100%]. Where indicated, MHC class I molecules of the target cells were blocked with 10 $\mu g/10^6$ cells of the mAb W6/32 (Serotec, Oxford, United Kingdom).

***Trans*-well Cultures of DCs, Apoptotic Tumor Cells, and NK Cells.** Tissue culture inserts with a pore size of 0.3 μm (Nunc) were used to create two compartments within culture plates to separate NK cells, DCs, or apoptotic Panc-1 tumor cells from the other cell types. Anti-IL-12 p40/p70 (clone C11.5, 30 μg/ml) and anti-IL-15 (clone 34505.11, 50 μg/ml) mAb (R&D Systems) were used as indicated. After 48 h, NK cell activation was assessed by intracellular IFN-γ staining as described above.

Statistical Analysis. Data are expressed as means ± SE. Statistical significance was determined by the Student *t* test for paired samples of original values. Differences were considered statistically significant for $P < 0.05$.

RESULTS

DCs Internalize Constituents of Apoptotic Tumor Cells. Apoptosis of Panc-1 tumor cells was induced by UV-B light or hyperthermia as assessed by annexin V and PI staining (Fig. 1*A*). After 24–48 h, when most tumor cells were early apoptotic (annexin V positive and PI negative), apoptotic tumor cells and their supernatants were separated and used for additional experiments. To study uptake of apoptotic tumor cells or released particles by immature DCs, tumor cells were stained with CFSE before the induction of apoptosis. PHK-26-PE-stained DCs were incubated with apoptotic tumor cells or their supernatants and analyzed by flow cytometry. Supernatant-pulsed DCs showed an increase of fluorescence intensity in the CFSE channel, indicating uptake of tumor-derived particles (Fig. 1*B*, *left graph*). To determine the capacity of DCs to capture whole apoptotic tumor cells, DCs were mixed with tumor cells in equal numbers. Double-positive cells represented DCs engulfing tumor cells (Fig. 1*B*, *right graph*). Intracellular uptake of tumor cells was confirmed by fluorescence and confocal microscopy (Fig. 1*C*). Neither apoptotic tumor cells nor their supernatants were toxic to DCs or induced maturation as assessed by PI staining and the lack of CD83 expression, respectively (data not shown).

Antigen-loaded DCs Induce a Th1 Cytokine Profile in Cocultures with PBMCs. Immature DCs were pulsed with lysate, apoptotic tumor cells, or their supernatants and were matured in the presence of TNF-α and PGE_2. After 24 h, the DCs expressed high levels of costimulatory molecules and the maturation marker CD83. These DCs were cocultured subsequently with autologeous PBMCs at a ratio of 1:20 in the presence of low doses of IL-2 and IL-7, and new DCs were added every 7 days. IFN-γ was detected in increasing concentrations after each stimulation in the culture medium of cocultures of PBMCs with antigen-pulsed DCs but not in the cultures with unpulsed DCs or PBMCs without DCs. When the different antigen preparations were compared, the highest amount of IFN-γ was found when apoptotic tumor cells were used as the source of antigen with no significant difference between UV-B- and hyperthermia-induced apoptosis (Fig. 2). The supernatant of apoptotic tumor cells induced less IFN-γ but significantly more than tumor lysate ($P < 0.01$ for heat- and $P = 0.02$ for UV-B-induced apoptosis). IL-4 and IL-10 were below the detection limit under any of these culture conditions (data not shown).

DCs Loaded with Tumor Antigens Elicit CTLs Able to Kill Pancreatic Carcinoma Cells. After four stimulations with DCs, the PBMCs were tested for their lytic activity against Panc-1 tumor cells in a ^{51}Cr-release assay. The lytic activity correlated with the amount of IFN-γ found in the culture medium, the highest tumor lysis being detected when apoptotic tumor cells were used for DC pulsing followed by supernatant and lysate (Fig. 3). Again, no statistical difference could be seen between UV-B- and heat-treated tumor cells. Supernatant of apoptotic tumor cells induced higher cell lysis rates than lysate. The difference was statistically significant for hyper-

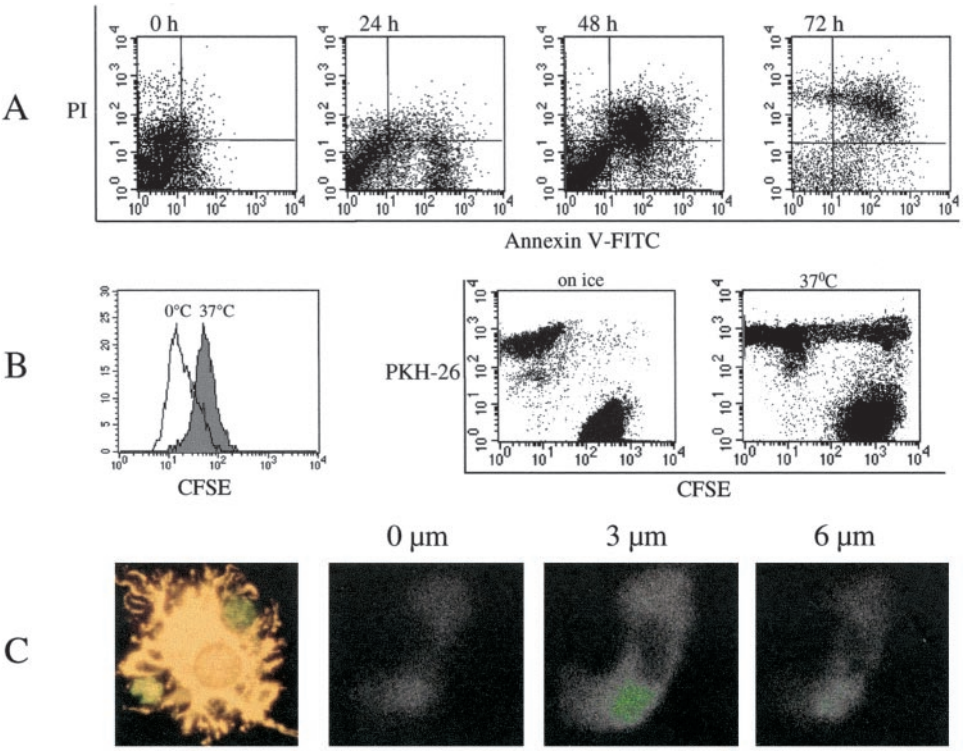

Fig. 1. Immature DCs internalize apoptotic Panc-1 tumor cells and released particles. Annexin V-FITC and PI staining show apoptosis of tumor cells after exposure to UV-B light (*A*). DCs capture constituents of apoptotic tumor cells (*B*). CFSE-stained apoptotic tumor cells (*dotplots*) or their supernatants (*histogram*) were incubated with PHK-26-stained DCs at a ratio of 1:1 for 4 h and analyzed by flow cytometry. Controls were incubated on ice. Internalization of tumor cells was analyzed by fluorescence and confocal microscopy (*C*). From *left* to *right*, a DC engulfs a tumor cell fragment (*green*, *top right*) 30 min after coincubation. Three confocal plains confirm intracellular position of a tumor cell fragment (*green*).

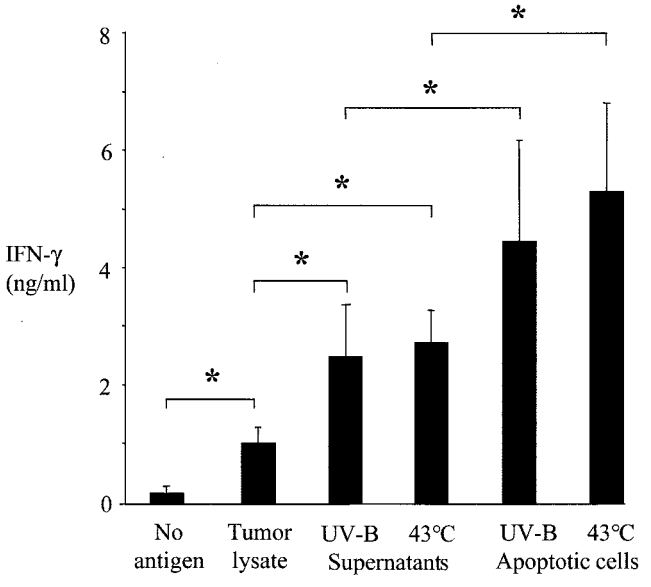

Fig. 2. IFN-γ production by PBMCs cocultured with tumor antigen-loaded DCs. DCs were pulsed with tumor lysate, supernatants of apoptotic tumor cells, or whole apoptotic tumor cells. After maturation with TNF-α and PGE_2, the DCs were cocultured with autologeous nonadherent PBMCs. The graph shows concentrations of IFN-γ in the supernatants of the PBMCs after the fourth weekly stimulation with DCs (mean ± SE, $n = 6$).

thermia-induced apoptosis ($P < 0.01$) but not for UV-B-induced apoptosis ($P = 0.09$). To determine the involvement of tumor antigen-specific CTLs in tumor killing, the experiments were also performed in the presence of the MHC class I-blocking antibody W6/32. Tumor cell lysis was effectively blocked in the experiments with antigens from cell lysate or tumor cell supernatant indicative of CTL-mediated tumor cell killing (Fig. 3). If CTL-mediated killing was defined as the difference between lysis without and with MHC I blocking, apoptotic tumor cell supernatants as antigen source were significantly superior to lysate ($P < 0.01$). Interestingly, PBMCs of the cocultures with apoptotic tumor cell-pulsed DCs still effectively killed tumor cells after MHC I blocking. Moreover, these cells were capable of lysing the unrelated gastric carcinoma cell line Kato-III, as well as the NK cell-sensitive cell line K562 (Fig. 4).

Apoptotic Tumor Cells Induce Activation of NK and γδ T Cells. To determine the activation pattern of different leukocyte subsets from PBMCs in the cocultures, we analyzed IFN-γ production of

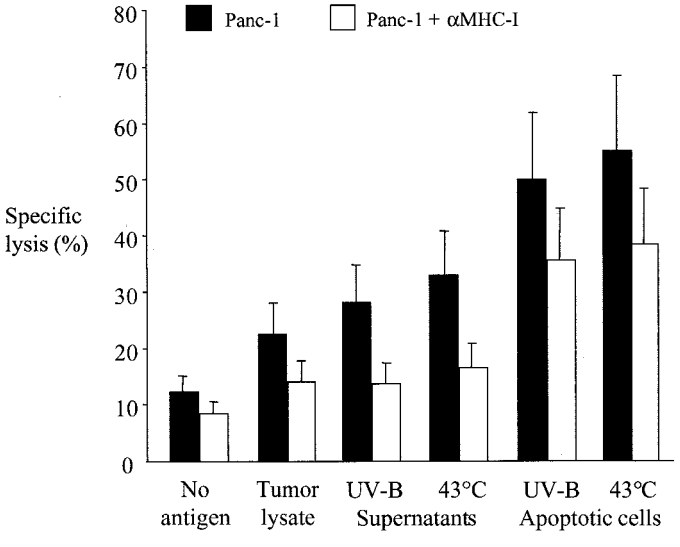

Fig. 3. Tumor cell killing by PBMCs stimulated with tumor antigen-pulsed DCs. DCs were pulsed with tumor cell lysate, supernatants of apoptotic tumor cells, or whole apoptotic tumor cells. Subsequently, DCs were washed, activated with TNF-α and PGE_2, and cocultured with autologeous PBMCs. Two days after the fourth weekly stimulation with DCs, the lytic activity of the PBMCs was assessed using ^{51}Cr-labeled Panc-1 tumor cells as targets in a ^{51}Cr-release assay (*solid bars*). To determine MHC class I restriction of tumor cell killing, the target cells were preincubated with the MHC class I-blocking antibody W6/32 (*open bars*). The specific lysis at an E:T ratio of 80:1 is shown (mean ± SE, $n = 6$).

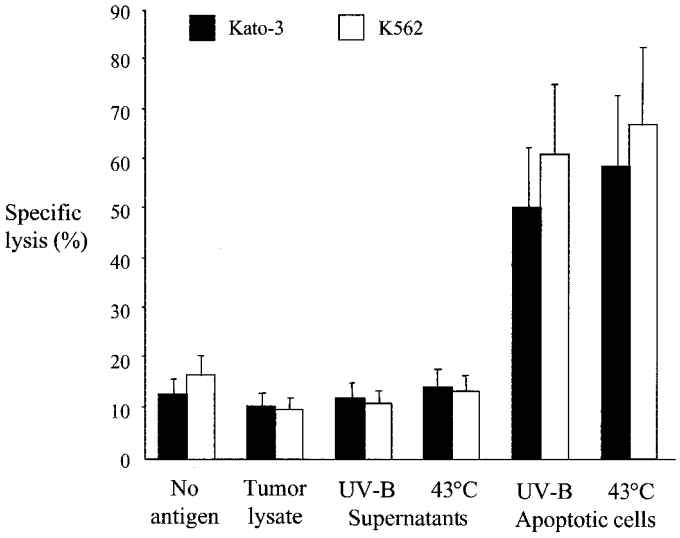

Fig. 4. PBMCs from cocultures with apoptotic tumor cell-pulsed DCs effectively kill unrelated tumor cell lines. After four weekly stimulations of PBMCs with DCs pulsed with different antigen preparations from Panc-1 tumor cells, the killing of the unrelated HLA-A2$^+$ gastric carcinoma cell line Kato-III ($n = 6$) and the NK-sensitive cell line K562 ($n = 5$) was assessed in a ^{51}Cr-release assay. The specific lysis at an E:T ratio of 80:1 is shown (mean ± SE).

CTLs (CD3$^+$ CD8$^+$), T helper cells (CD3$^+$ CD4$^+$), NK cells (CD3$^-$ CD56$^+$), and γδ T cells (CD3$^+$ Vγ9$^+$) by flow cytometry. As expected from the cytotoxicity assays, the activation pattern of leukocyte subsets was quite different if whole apoptotic tumor cells were chosen as the source of antigen compared with supernatant or lysate (Fig. 5). A portion (23% hyperthermia and 19% UV-B) of NK cells were activated in PBMCs cocultured with apoptotic tumor cell-pulsed DCs. Furthermore, 30% (hyperthermia) and 36% (UV-B) of γδ T cells stained positive for IFN-γ under the same conditions. In contrast, only a small number of NK and γδ T cells from cocultures with supernatant- or lysate-pulsed DCs produced IFN-γ. No significant differences in CTL activation could be seen between apoptotic tumor cell-pulsed *versus* supernatant-pulsed DCs. However, both conditions were clearly superior to lysate with a higher frequency of IFN-γ-producing CTLs and T-helper cells.

NK Cell Activation Is Dependent on Tumor Cell Contact and IL-12 Produced by DCs. Because tumor cells, which were not internalized by DCs, were still present in the cocultures of PBMCs with tumor cell-pulsed DCs, we were interested if direct tumor cell contact with NK cells was necessary for NK cell activation or if DC directly triggered NK cell activation. Activation of NK cells (purity > 95%) was studied by intracellular IFN-γ staining in a *trans*-well culture system allowing only direct contact between two of the three cell types: DCs and apoptotic tumor cells, NK cells and apoptotic tumor cells, or DCs and NK cells. Only in cultures that allowed direct cell-to-cell contact between NK cells and apoptotic tumor cells, NK cell activation was observed (Fig. 6). However, no NK cell activation was seen in the absence of DCs (data not shown). Therefore, most likely, a soluble factor secreted by DCs was involved. Adding IL-12 was able to substitute for DCs. Moreover, IL-12-blocking antibodies inhibited activation when DCs were present (Fig. 6), whereas blocking IL-15 showed no effect. This indicated that NK cell activation depended on both direct cell contact with tumor cells, as well as DC-derived IL-12.

DISCUSSION

Cross-priming of CTLs with antitumor activity has been demonstrated with DCs pulsed with tumor cell lysates (17), apoptotic tumor cells (18–22), and apoptotic bodies (23), but to our knowledge, no quantitative comparison has been undertaken. In this study, we investigated the ability of monocyte-derived DCs pulsed with different antigen preparations from the pancreatic carcinoma cell line Panc-1 to induce an antitumor T-cell response in a cross-presentation *in vitro* model. The antigen preparations consisted of: (*a*) tumor cell lysates; (*b*) UV-B- or hyperthermia-induced apoptotic tumor cells; or (*c*) their supernatants, containing low-density particles released from the cells, such as apoptotic bodies. Immature DCs internalized tumor cells, as well as released particles, as shown by flow cytometry and confocal microscopy. After antigen loading, we activated the DCs with cytokines to induce a T-cell stimulatory phenotype for effective T-cell priming.

Our results showed that antigens from apoptotic tumor cells, whole cells as well as released particles, were more potent than tumor lysates in inducing T-cell priming and activation by DCs. This was evidenced by enhanced IFN-γ secretion and a higher frequency of activated CTLs and T-helper cells, as well as a higher rate of MHC class I-restricted tumor cell killing. This result is in agreement with a report from Hoffman *et al.* (19), who observed stronger CTL responses with apoptotic tumor cells, compared with cell lysates in a squamous cell carcinoma model. Enhanced CTL activation by antigens from apoptotic cells may be attributed to several mechanisms. After internalization, most particulate antigens requiring phagocytosis are digested into peptides associating with MHC class-II molecules in the endocytic compartments and are presented to T-helper cells (27). This is believed to be the predominant processing pathway of cell lysates. In contrary, scavenger receptor-mediated phagocytosis of apoptotic tumor cells allows antigens to gain access to MHC class-I compartments, resulting in cross-presentation of the antigen to CTLs (18, 28). In addition, enhanced CTL responses against tumors might be mediated by hsps expressed by stress-induced apoptotic tumor cells (29). Hsps have been shown to improve uptake of antigens by DCs (30) and can provide additional antigenic epitopes by peptides complexed to hsp (31). On the basis of this theoretical background and our own observations, we conclude that antigen preparations from apoptotic

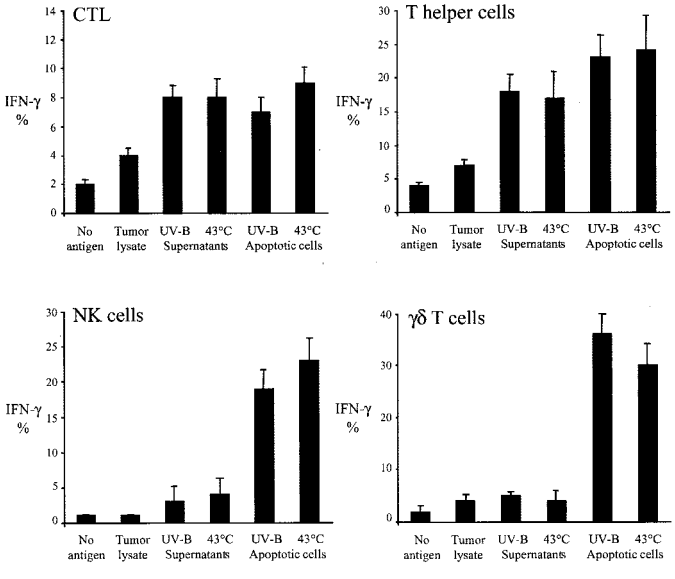

Fig. 5. Activation pattern of different lymphocyte subsets in PBMCs cocultured with tumor antigen-pulsed DCs. PBMCs were removed 12 h after the fourth stimulation with DCs, and intracellular IFN-γ was stained after incubation with brefeldin A. In a four-color flow cytometric analysis, CTLs were identified as CD3$^+$ CD8$^+$, T-helper cells as CD3$^+$ CD4$^+$, NK cells as CD3$^-$ CD56$^+$, and γδ T cells as CD3$^+$ v$_γ$9$^+$. Data represent means of percentage of IFN-γ-positive cells ± SE ($n = 5$).

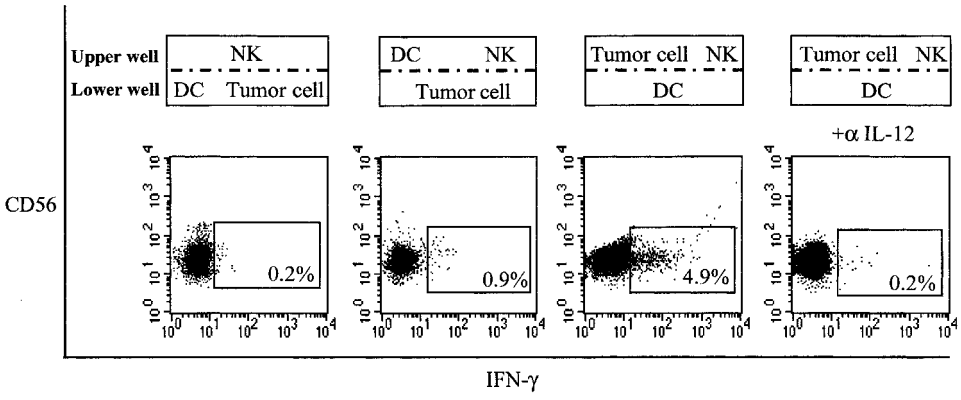

Fig. 6. DC-mediated activation of NK cells is dependent on direct cell-to-cell contact between apoptotic tumor cells and NK cells, as well as the presence of IL-12. Purified NK cells (*NK*) were cocultured with DCs and UV light-induced apoptotic tumor cells in a *trans*-well culture system. On day 2, NK cell activation was assessed by intracellular IFN-γ staining. The culture conditions are shown in the *top*. Where indicated, IL-12 (p40/p70)-blocking antibodies were added to the medium. A representative experiment of six is shown.

tumor cells represent a promising alternative to tumor lysate in DC-based tumor vaccines.

Tumor cell killing was highest when whole apoptotic tumor cells were used as the source of antigen. However, compared with experiments with supernatant or lysate, tumor cell killing was only partially MHC class I restricted. Moreover, unrelated cell lines were also effectively killed. The analysis of the activation pattern of different leukocyte subsets in these cocultures revealed not only activation of CTLs and T-helper cells but also of NK cells and γδ T cells. The latter two are effector cell types of the innate arm of the immune defense with the ability to kill tumor cells in an antigen-unspecific manner. In a recent study, vaccinating mice with DC cocultured with tumor cells induced CTL-mediated protective immunity, as well as NK cell activation (10). Interestingly, in that study, NK cell depletion abrogated tumor protection favoring the concept that combining innate and acquired effector mechanisms may enhance tumor immunogenicity.

Mechanisms leading to NK and γδ T-cell activation by tumor cells are only understood incompletely. Activation seems to be influenced by a balance of negative and positive signals. A positive signal can be mediated by the NKG2D-DAP10 receptor complex, expressed on the surface of NK cells and γδ T cells, which is known to interact with stress-induced ligands on tumor cells, such as MICA and Rae-1 (32–35). Stimulation via NKG2D can override negative signals from inhibitory receptors and triggers degranulation and perforin-mediated apoptosis of the tumor cell. Another concept favors that direct cell-to-cell contact between DCs and NK cells enhances the cytolytic activity of NK cells (7). In our experiments, apoptotic tumor cells were present in cocultures of PBMCs with apoptotic tumor cell-pulsed DCs. This was not the case in the cocultures with supernatant- or lysate-pulsed DCs, because excess antigen was removed by washing. Therefore, it seemed likely that cell contacts of tumor cells with NK cells within the PBMCs played a role in the observed NK cell activation. When we analyzed the required components for this effect in a *trans*-well cultures system, we found that direct cell contact between NK cells and apoptotic tumor cells was required, as well as a soluble factor produced by DCs. A recent report also points into this direction, but the factor was not identified (36). In cytokine-blocking experiments, we found that NK cell activation was dependent on DC-derived IL-12. Therefore, DCs played a dual role in these cultures: (*a*) they cross-presented tumor antigen to T cells inducing tumor-specific CTLs; and (*b*) they activated innate effector cells via IL-12. A vaccination trial using DCs fused with tumor cells, achieved promising results in patients with renal cell carcinoma (4). Because in that study fusion was only effective partially, unfused tumor cells were injected together with DCs; possibly, activation of innate effector cells by direct contact with stressed tumor cells in the presence of DC-derived IL-12 played a role in the vaccination success observed in some patients.

In conclusion, pulsing DCs with antigen from apoptotic tumor cells seems to be a promising alternative to lysate and might be used for tumors when immunogenic epitopes are unknown. Furthermore, a vaccine containing apoptotic tumor cells in addition to DCs could offer advantages by stimulating effector cells of the innate immune system. Activation of NK cells and γδ T cells at the injection site could provide stimuli for local antigen-presenting cells, enhancing the effectiveness of the vaccine. However, the efficacy of this strategy needs further evaluation.

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Distribution and Maturity of Dendritic Cells in Diseases of Insufficient Placentation

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Problem
The immunological equilibrium at the feto-maternal interphase contributes towards late gestational diseases like growth restriction (IUGR) pre-eclampsia (PE) and hemolysis, elevated liver enzymes, low platelets (HELLP)-syndrome. The state of activation of decidual dendritic cells (DC) has emerged as one of the central players influencing this immunological equilibrium.

Method of study
Paraffin-embedded tissue sections from 27 pregnancies were immunostained for DC markers DEC-205, DC-SIGN, DC-LAMP and costained for DC-SIGN/CD56 and DC-SIGN/ vascular endothelial growth factor receptor (VEGFR) -1 and -2. We investigated placental tissue of IUGR fetuses and of patients who developed PE or HELLP-syndrome as well as placental tissue derived from normal pregnancies.

Results
We found that expression of DEC-205 and DC-SIGN was significantly upregulated in HELLP placentas, whereas expression of DC-LAMP was abrogated almost entirely. Costaining showed an interaction between DC-SIGN$^+$ DC and natural killer cells as well as costaining of VEGFR-1 and -2 and DC-SIGN. Pre-eclamptic and IUGR placentas showed no significant change in any of the investigated markers compared to normal controls.

Conclusion
Our data suggest a participation of DC-mediated immunological mechanisms in HELLP syndrome.

Introduction

Mammalian reproduction has to overcome unique immunological challenges, as the semiallogeneic blastocyst expresses alloantigens inherited from the father. The growing fetus is not merely tolerated by the maternal immune system during pregnancy. There is growing evidence that the immunological equilibrium at the feto-maternal interphase plays an integral part during placentation.[1] Hypertensive diseases of pregnancy have been associated with immune phenomena.[2,3] The notion exists that that trophoblast invasion is disturbed by a dysfunctional maternal immune reaction, resulting in incomplete spiral artery transformation with subsequent placental insufficiency and feto-maternal compromise.[4] Dendritic cells (DC) have long been known to be central regulators of adaptive immunity.[5,6] Recent

research, however, points toward a role of DC in maintaining peripheral tolerance.[7] Accumulating data show that DC contribute significantly towards mucosal tolerance to exogenous antigens in the skin, lung, and gut.[8–10] The concept of DC as the 'gatekeepers of mucosal immunity' has recently been extended to uterine mucosal immunity and its immunological relationship to the semi-allogeneic conceptus.[11] The state of activation of DC determines their immune-stimulatory capacities and therefore might contribute toward optimal decidual immune responses supporting placento-fetal development.[12]

In the reported experiments, we assessed immune-histochemical maturation markers of decidual DC in placental tissue of pregnancies complicated by pre-eclampsia (PE) (*de novo* hypertension, and proteinuria) beyond 20 weeks of gestation, HELLP-syndrome (hemolysis, elevated liver enzymes, low platelets) and intrauterine growth restriction (IUGR).

Materials and methods

Tissue Samples

Placental tissues were obtained from 27 women who had given birth in the first Department of Obstetrics and Gynaecology of the LMU Munich. Six placentas with IUGR (mean date of delivery ± S.D.: 33 ± 3 weeks of gestation), eight placentas with PE (mean date of delivery: 33 ± 3.2 weeks of gestation), five placentas with HELLP (mean date of delivery: 33.8 ± 2.9 weeks of gestation) and eight normal placentas (mean date of delivery: 38.2 ± 3 weeks of gestation) were used for the study (see supplementary data Table S1 for fetal clinical outcome).

Fetal intrauterine growth restriction was defined as an estimated intrauterine weight below the fifth centile after gestational age had been confirmed by an early pregnancy ultrasound. IUGR needed to be confirmed after delivery; otherwise the patient was excluded from the study.

Pre-eclampsia was defined as maternal hypertension >140/90 mmHg without previous hypertensive history and 300 mg/L proteinuria without any history of renal disease.

Patients with HELLP-syndrome included in the study had a minimum of twofold increased liver enzymes, platelets below 100,000/lL and detectable haptoglobin below the normal range. The study was approved by the ethics committee of the Faculty of the Ludwig-Maximilians-University Munich, Germany, and informed consent was obtained from each patient.

Placental samples were taken at three central parts of the placenta. Obtained tissue was frozen immediately after delivery and stored at −20°C. A second set of placental tissue was fixed immediately after delivery in 4% buffered formalin for 20–24 hr and embedded in paraffin afterwards.

Immunohistochemistry

All paraffin-embedded specimens were cut to 2–3 lm and mounted on SuperFrost/Plus microscope slides (Menzel, Walldorf, Germany). After deparaffinization and rehydration, slides of different placental tissue specimens were incubated in methanol/H_2O_2 (30 min) to inhibit endogenous peroxidase activity. This was followed by washing in phosphate-buffered saline (PBS, pH 7.4) (5 min) and treatment with rabbit or horse serum (20 min, 22°C, Vector laboratories, Burlingame, CA, USA) to reduce non-specific background staining. There was no pre-treatment of the slides. Tissues were incubated with the primary antibodies (see supplementary data Table S2) for 1 hr at room temperature. Sections were incubated with the biotinylated secondary antibody (1 hr, 22°C, Vector laboratories) and avidin-biotinylated peroxidase (45 min, room temperature, Vector laboratories). In between each step, sections were washed with PBS. Peroxidase staining reaction was performed with diaminobenzidine/H_2O_2 (1 mg/mL; 5 min, Vector laboratories) and stopped in tap water (10 min). For double immune-histochemistry, we used secondary antibodies conjugated with fluorescein-5-isothiocyanate or phycoerythrin (PE) conjugated mAb (Dako, Ely, Cambridge, UK). The slides were finally embedded in mounting buffer containing 4′,6-diamino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA). Single stain sections were counterstained in hemalaun (1 min, Vector laboratories) and then cover-slipped. In controls, the primary antibody was replaced with pre-immune mouse serum with positive and negative controls included. All specimens were evaluated by a pathologist (D.M.). The intensity and distribution patterns of the staining reaction were evaluated by two independent observers, including a gynecological pathologist (D.M.), using the semi-quantitative immunoreactive score (IRS), as previously described,[13] and used to assess steroid receptors [14]

and cathepsin D [15] expression in human endometrial tissue. The IRS was calculated by multiplication of optical staining intensity (graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positive stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = >81% of the cells). Evaluation of each specimen was performed without having any knowledge of the clinical diagnosis.

Statistics

The spss/pc software package version 15.0 was used for collection, processing, and statistical data analysis. Statistical analysis was performed by using non-parametric Wilcoxon test for matched pairs and non-parametrical Mann–Whitney U-test for comparison of the means. $P < 0.05$ values were considered statistically significant.

Results

Dendritic cells in the human decidua were analyzed by determining five times the number of cells per field of view with a 25× lens magnification. We investigated DC density and state of activation in all collected samples.

Density and Maturation of Decidual Dendritic Cells

In decidua of normal term placentas, a median number of five DC expressing DC-SIGN was seen. Compared to normal term placentas, a significant upregulation of DC expressing DC-SIGN was determined in the decidua of HELLP placentas ($P = 0.002$) with a median of 18, whereas DC-SIGN was downregulated non-significantly in IUGR patients ($P = 0.061$). No significant changes were seen in pre-eclamptic decidual tissue compared to normal control placentas [($P = 0.255$); median of four DC-SIGN$^+$ cells per field of view). The expression of DC-SIGN in normal, HELLP and IUGR placentas is shown in Fig. 1a–c. Fig. 1d summarizes these results.

The median of decidual DC staining for DEC-205 in control placentas was four cells per field of view (Fig. 2a). Staining for DEC-205 was significantly increased in HELLP placentas ($P = 0.001$) with a median density of 10, whereas IUGR placentas showed a lower expression ($P = 0.052$; median = 3) (Fig. 2b,c). In pre-eclamptic placentas, we saw a non-significantly reduced amount of DC staining positive for DEC-205 ($P = 0.226$; median = 3) as compared to normal placentas. These results are shown in Fig. 2d.

In decidual tissue of normal term control placentas, DC positive for DC-LAMP had a rather high density with a median of 19.5 (Fig. 3c). We

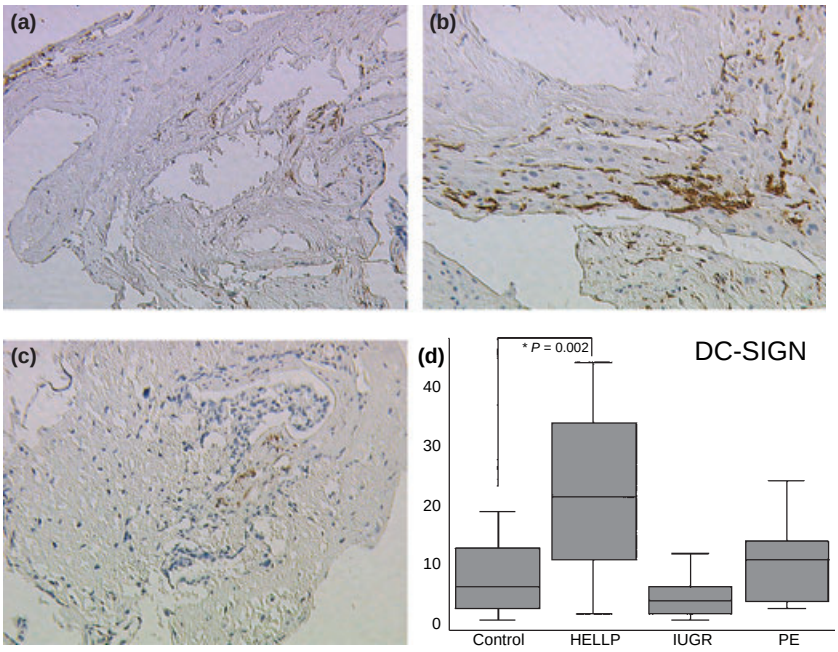

Fig. 1 Expression of DC-SIGN in normal (a), HELLP (b) and IUGR (c) placentas, all pictures 10x lens. A summary of staining results is presented in 1 (d).

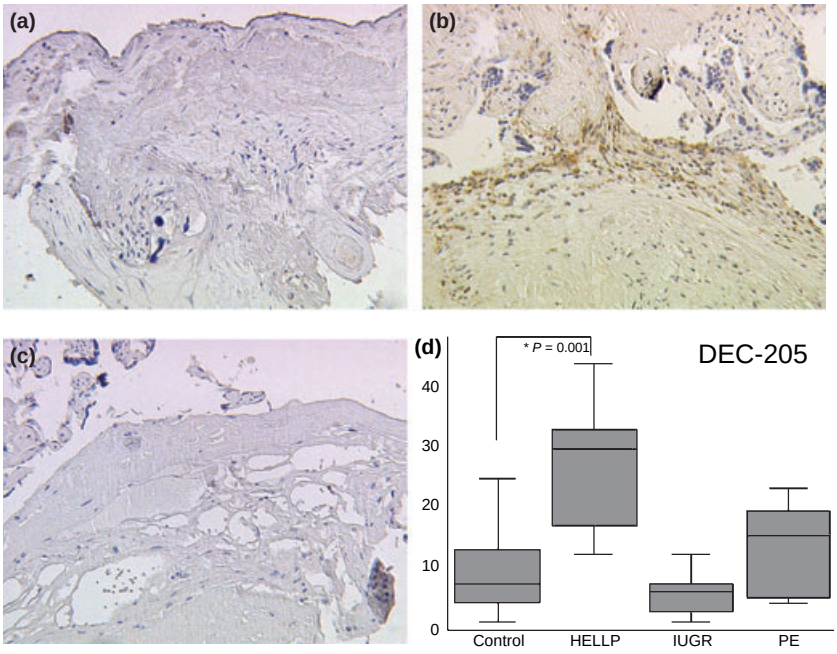

Fig. 2 Expression of DEC-205 in normal (a) HELLP (b) and IUGR (c) placentas, all pictures 10x lens. A summary of staining results is presented in 2 (d).

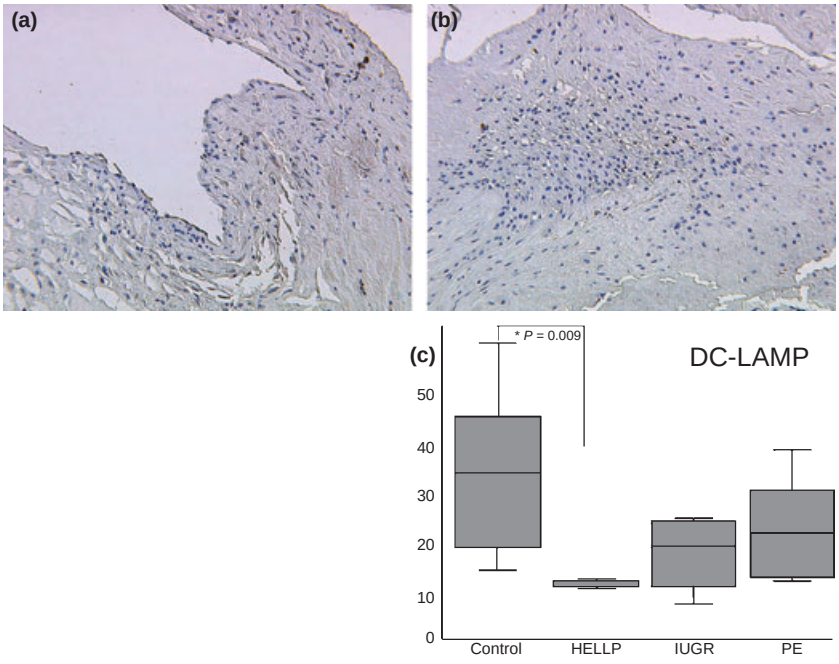

Fig. 3 Expression of DC-LAMP in normal (a) and HELLP (b) placentas, both pictures 10x lens. A summary of staining results is presented in 3 (c).

noted a complete abrogation of DC-LAMP in HELLP patients (P = 0.009) (Fig. 3b). There was no significant decrease of DC-LAMP in IUGR (P = 0.109; median = 7) as well as PE placentas (P = 0.301; median = 16.5). These findings are summarized in Fig. 3c. A representative serial section stained for the different DC markers is shown in Fig. 4.

Functional Properties of Decidual Dendritic Cells and Natural Killer (NK) Cells and Their Interaction

We encountered a high proportion of DC-SIGN positive DC in HELLP placentas and examined whether these cells colocalized with NK-cells at the feto-maternal interphase. We saw a distinctive

decidual zone of interaction between DC-SIGN$^+$ DC and CD56$^+$ NK-cells that was visible exclusively in HELLP placentas and was completely absent in normal controls (Fig. 5). DC-SIGN$^+$ DC in HELLP placentas also expressed vascular endothelial cell growth factor receptor (VEGFR) -1 & -2. For VEGFR-2, we saw strong costaining in the immediate proximity of sections of decidual vasculature. A pattern that was less prominent for VEGFR-1 (data not shown). In normal controls, VEGFR-2 was expressed, but did not costain with DC-SIGN (Fig. 5).

Taken together, we encountered a different pattern of distribution and state of activation at the feto-maternal interphase of pregnancies with HELLP syndrome compared to normal pregnancies as well as pregnancies complicated by IUGR or PE. Furthermore, we saw an interaction zone between NK-cells and DC-SIGN$^+$ DC exclusively in HELLP placentas. In these placentas, DC-SIGN$^+$ DC costained for VEGFR, in contrast to normal controls.

Discussion

An immunological contribution towards placental gestational diseases has been suspected for many years. We investigated the role of DC within the immunological equilibrium at the feto-maternal interphase. To asses DC density and state of maturation, we chose three characteristic DC surface molecules: The C-type lectin-receptor DEC-205 is fairly ubiquitously expressed on immature DC and is mildly upregulated upon maturation.[16] DC-SIGN, from the same macrophage mannose receptor family of C-type lectins, is a molecule that is specifically expressed on immature DC. Its expression, however, is downregulated upon maturation.[17] DC-SIGN has also been put forward as a putative functional player in immunological pregnancy recognition in rhesus macaques.[18] The third marker DC-LAMP constitutes a surrogate marker for mature, immunogenic DC.[19] In this study, histochemical staining showed no significant changes in DC maturation markers in pre-eclamptic and growth restricted pregnancies compared to normal controls. However, patients who developed HELLP syndrome showed a markedly different DC morphology in their placentas. We detected more decidual DC that stained almost entirely positive for DC-SIGN, and therefore exhibited an immature phenotype. This result is in concordance with data showing a high number of proliferating DC-SIGN positive DC in early human pregnancy.[20] Although the extent to which normal first-trimester decidual DC express DC-SIGN is under scientific dispute, there is consensus regarding DEC-205 as a decidual DC-marker.[12] To the best of our knowledge, our results represent the first data showing the expression of the unique DC maturation marker DC-LAMP in human decidua. The lysosome-associated membrane glycoprotein, DC-LAMP has been used to characterize mature DC in human cancerous tissue and is usually expressed upon encounter of danger signals.[21,22]

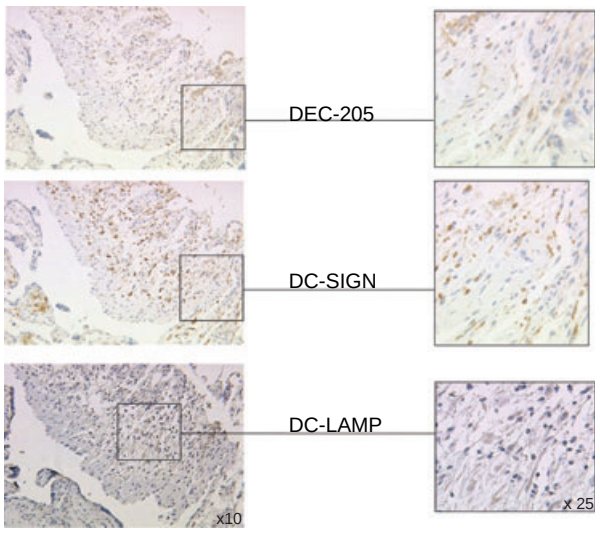

Fig. 4 Serial section of a representative HELLP placenta stained for DEC-205, DC-SIGN and DC-LAMP. Left column 10× magnification. Right column 25× magnification.

HELLP syndrome has been considered in some parts as a variant of PE. Its pathophysiology remains elusive, although an immunological contribution is suspected.[3] A systemic immune dysbalance with an emphasis on T helper pathways has been described for PE.[23,24] In this study, we are able to demonstrate a significant increase in DC-SIGN$^+$ DC and decrease in DC-LAMP$^+$ DC restricted to HELLP syndrome derived placentas. PE derived placentas did not show any significant difference in the maturation pattern of DC. We speculate that this DC-SIGN^{high}, DC-LAMP^{low} pattern of decidual DC might be due to an increased apoptotic activity in HELLP syndrome. Pre-eclampsia, as well as HELLP-syndrome, is known to exhibit increased apoptosis of trophoblastic tissue, although to a different degree.[25] Without further maturation stimuli, apoptotic cell material is known to skew DC-maturation processes into a tolerogenic

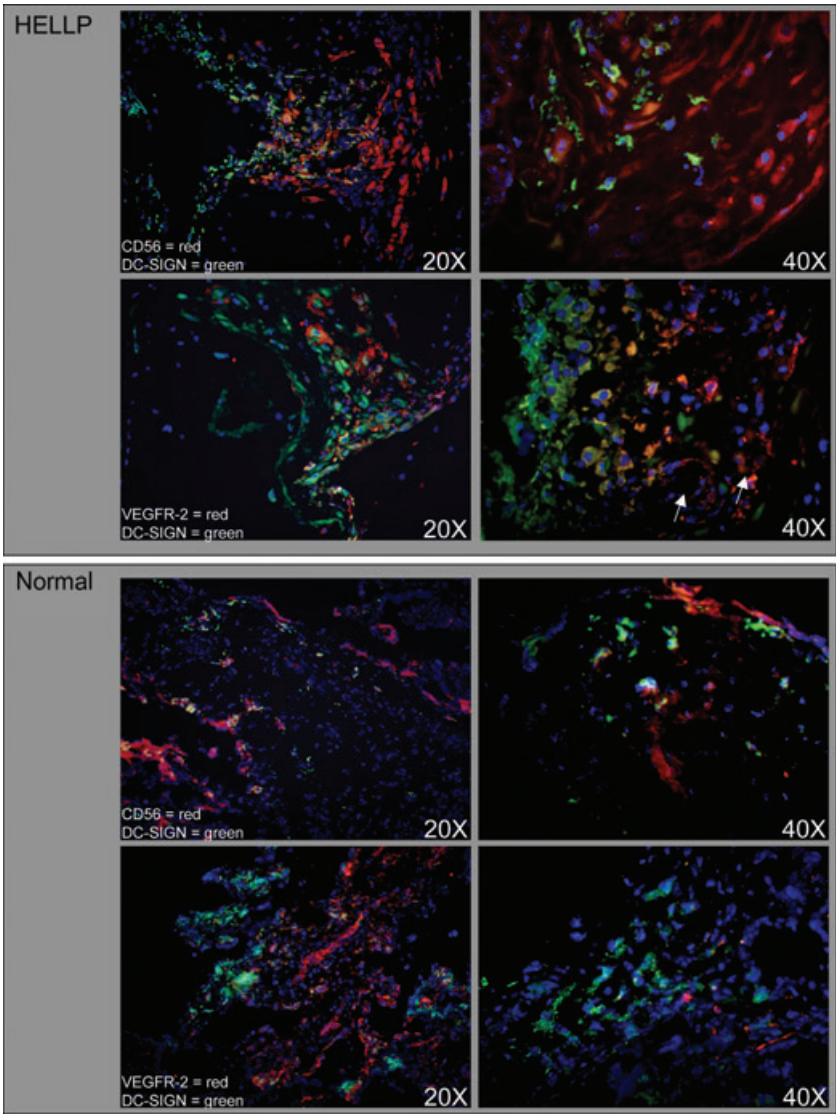

Fig. 5 Double staining of DC-SIGN (green) and CD56, VEGFR-2 (red) respectively. Upper panel shows costains of one representative HELLP placenta at the feto-maternal interphase as compared to one representative normal control (lower panel). Higher magnification (40×) of the same placenta is provided in the right column. Arrows indicate cross sections of maternal blood vessels.

phenotype and foster cross-priming of exogenous cell material by DC.[26,27] There is accumulating evidence that there is an intensive cross talk between DC and NK-cells in which lytic behavior and cytokine secretion of NK-cells are influenced by the activation pattern of DC.[11,28] Our morphological data suggest an intimate relationship between DC and NK-cells in HELLP syndrome. Uterine NK-cells are known to play a central role in placentation ensuring proper vascularization by secretion of an array of angiogenic factors and by inducing vascular growth in the decidua.[29] The close interaction we observed in HELLP placentas might therefore reflect a compensatory immunological process to foster vascularization of an otherwise underperfused placenta. A contribution of DC towards vascularization in HELLP-syndrome was also seen for VEGFR1&2 as these receptors were coexpressed by a subset of DC-SIGN^{+} DC in HELLP syndrome decidua. In tumorigenesis, DC contribute to neovascularization via secretion of angiogenic factors.[30,31] Increased sVEGFR-1&-2 secretion has been shown for placental tissues from pregnancies complicated by hypertensive disorders.[32] Decidual DC display a distinct state of activation and our data support evidence that alternatively activated DC play a role within angiogenetic networks that are altered in insufficient placentation.[33] Taken together, we encountered a DC-SIGN^{high}, DC-LAMP^{low} phenotype at the feto-maternal interphase of HELLP patients, that colocal-

ized with $CD56^+$ NK-cells and costained for VEGFR. Whether this constitutes a cause or consequence of a dysfunctional immune response in these patients might be difficult to distinguish. Our data suggest a contribution of decidual DC within this immune response and might stimulate further studies elucidating their interaction with other immune cells at the feto-maternal interphase.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1 Clinical details on the patients and newborns in the HELLP, IUGR and normal control group.

Table S2 Antibodies used in the study.

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Estrogen receptor modulators and estrogen receptor beta immunolabelling in human umbilical vein endothelial cells

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Human umbilical vein endothelial cells;
Estrogen receptor;
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Estrogen receptor antagonist

Summary

Human umbilical vein endothelial cells (HUVEC) exposed to the female sex hormone estradiol show different kinds of effects including increased elasticity, activation of plasma membrane Na^+/H^+ exchange, prostacyclin production, prevention of apoptosis and many others. The aim of this study was the systematic analysis of the immunolabelling of estrogen receptors (ERs), ERa and ERb, in HUVEC after stimulation with different commercially available ER modulators and ER agonists or antagonists. HUVEC response to these substances was shown to be regulated via ERb. ERa immunolabelling or up-regulation was abrogated after application of estrogen derivatives, selective estrogen receptor modulators (SERM) and ER agonists or antagonists. Immunolabelling of ERb was significantly increased by estradiol, estrone, ethinylestradiol and tumour necrosis factor alpha (TNFa). SERM, such as Tamoxifen, and pure antagonists, such as ICI 182.780, stimulated ERb in HUVEC at low concentrations, whereas higher concentrations inhibited ERb immunolabelling. The pure estrogen receptor agonist 2,3-bis (4-hydroxyphenyl) proprionitrile (DPN) exhibited its activating potential at low concentrations. In contrast, higher concentrations resulted in a down-regulation of ERb. Estrogenic effects in HUVEC, independent of stimulation or inhibition, are mediated via the ERb. SERM such as Tamoxifen and ER antagonists such as ICI 182.780 act as ER activators in low concentrations, whereas higher concentrations lead to inhibitory effects.

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Introduction

Human endothelial cells are involved in a diversity of pathophysiological processes, including hemostasis, inflammation and angiogenesis (Park et al., 2006). Estradiol (E2) and its metabolites are known to delay the onset of atherosclerosis in women compared to men (Hillebrand et al., 2006). However, the cellular mechanisms of these gender-specific differences remain unclear. Human umbilical vein endothelial cells (HUVEC) serve as a widely used in vitro model to study the effects of natural and synthetic female sex hormones on endothelial cells.

The female sex hormone E2 exerts its actions on target cells through binding and activation of the estrogen receptor (ER), a member of the steroid hormone superfamily of ligand-dependent transcription factors. Estradiol and its metabolites bind to control regions (promoters) of specific gene elements via receptor–ligand complexes, recruit co-activators/-repressors and control gene expression depending on the kinetics of administration (Brosens et al., 2004; Simoncini et al., 2005). Furthermore, estrogenic effects on volume, growth and elasticity of HUVEC (Morales et al., 1995; Hillebrand et al., 2006), as well as the release of activation markers such as prostacyclin (Hermenegildo et al., 2005; Oviedo et al., 2005), nitric oxide (NO) (Florian et al., 2004), interleukins (Keck et al., 1998), vascular cell adhesion molecule-1 (Mukherjee et al., 2002; Tatsumi et al., 2002) and others, (Akarasereenont et al., 2000; Chen et al., 2002) have been demonstrated.

There is an ongoing discussion as to whether estrogenic effects are mediated through ERa, ERb or both. Although Jensen et al. (1998) and Tatsumi et al. (2002) showed that HUVEC do not express the classical ERa, other investigators including Herve et al. (2006); Evans et al. (2002) demonstrated weak immunolocalisation of both ERa and ERb mRNA in HUVEC. A diversity of ER activators and inhibitors exist, each with different potential to bind to ERa, ERb, or both. The aim of this study was to analyze the amount of immunolabelling of ERa and ERb in HUVEC after stimulation with estrogen derivatives, selective estrogen receptor modulators (SERM), ER agonists and antagonists using specific monoclonal antibodies and immunocytochemistry.

Material and methods

Cell culture

HUVEC were obtained from Promocell (Heidelberg, Germany) at passage 2 or 3. The cells were cultivated in phenol red-free endothelial cell growth medium (ECGM) (Customer formulation, Promocell, Heidelberg, Germany) containing 10% fetal bovine serum, 1.0 mg/ml hydrocortisone, 0.1 ng/ml endothelial growth factor, 1.0 ng/ml basic fibroblast growth factor (bFGF). About 2 ml endothelial cell growth substrate, 5 ml streptomycin (Biochrom AG, Berlin, Germany) and 5 ml amphotericin B (Biochrom AG, Berlin, Germany) were added per 500 ml of medium.

HUVEC were used for experiments between passages 3 and 7. All cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37 1C. HUVEC were incubated with E2, estrone, ethinylestradiol (EE), Tamoxifen, ICI 182.780 and 2,3-bis (4-hydroxyphenyl) proprionitrile (DPN) as well as tumour necrosis factor alpha (TNFa) in different concentrations (0–2000 nmol/ml) (Table 1) and cultivated for up to 72 h on chamber slides (Quadripermm Nunc, Wiesbaden, Germany). These concentrations are related to physiological estrogen concentrations in pregnant human females. Cells were fixed with methanol/ethanol (50%/50%) (Merck, Darmstadt, Germany). Chamber slides were then dried, wrapped and stored at −80 1C as described by Toth et al. (2008) until required. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study.

Table 1. Substances used for HUVEC stimulation

Substance	Concentrations	Source
17b-Estradiol	10, 1, 0.1, 0.01, 0.001 nmol/ml	Sigma-Aldrich, Munich, Germany
Ethinylestradiol (EE)	1800, 1000, 500, 100, 10, 1, 0.1 nmol/ml	Jenapharm, Jena, Germany
Estrone	1000, 500, 100, 10, 1, 0.1 nmol/ml	Sigma-Aldrich, Munich, Germany
DPN	500, 100, 10, 1, 0.1 nmol/ml	Tocris, Bristol, UK
ICI 182,780 = Fulvestrant	500, 100, 10, 1, 0.1 nmol/ml	Tocris, Bristol, UK
Tamoxifen	500, 100, 10, 1, 0.1 nmol/ml	Sigma-Aldrich, Munich, Germany
TNFa	10, 1, 0.1 ng/ml	Sigma-Aldrich, Munich, Germany

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Immunocytochemistry

Immunolocalisation of ERa and ERb was analyzed by using specific monoclonal antibodies and an avidin biotin complex (ABC) labelling kit (Vectastain Elite mouse-IgG-Kit, Vector, Burlingame CA, USA) employed according to manufacturer's instructions. All reagents are from this kit unless otherwise specified. In this study we used monoclonal antibodies against ERb (Clone: PPG5/10, Isotype: mouse IgG2a, dilution: 1:600, Serotec, Düsseldorf, Germany) and ERa (Clone: ER1D5, Isotype: mouse IgG1, dilution: 1:50, Immunotech, Prague, Czech Republic).

After thawing, cells were briefly fixed with formalin (Merck, Darmstadt, Germany; 5% in phosphate buffered saline (PBS), 5 min). Slides were incubated in 3% methanol/H_2O_2 for 30 min to inhibit endogenous peroxidase activity and treated with 1.5% goat serum/PBS for 20 min to reduce non-specific background staining. Incubation with the primary antibody was performed overnight at 4 1C. Sections were then incubated with biotinylated secondary anti-mouse antibody (1:200) for 1 h and then with avidin biotin peroxidase complex (1:25) (ABC) prepared according to manufacturer's instructions for 45 min (Vectastain-Elite-ABC Kit, Vector Laboratories, Burlingame CA, USA). Labelling was revealed by incubation with 1 mg/ml diaminobenzidine/H_2O_2 (Dako, Hamburg, Germany) for 5 min and the reaction stopped by washing in tap water for 10 min. Sections were counterstained in hematoxylin for 1 min, rinsed in tap water for 5 min followed by distilled water and then coverslipped in Aquatex (Merck, Darmstadt, Germany). All steps were performed at room temperature and all dilutions and thorough washes between stages were performed using PBS unless otherwise stated. In negative controls, the primary antibody was replaced with pre-immune mouse serum. Labelling was performed in a single run with identical staff, equipment and chemicals, and a total of six independent experiments were performed ($n = 6$). Positive and negative controls were always included. As a positive control we used the ER-expressing breast cancer cell line MCF7. The slides were finally mounted in Aquatex mounting media (Merck, Darmstadt, Germany) and examined using a Zeiss Axiophot photomicroscope (Carl Zeiss, Jena, Germany). From each section, five digital pictures were captured randomly at 200-fold magnification using a 3CCD color camera (Axiocam). Labelling intensity was assessed using a semi-quantitative score by counting the absolute percentage of positively labeled cells. Two independent observers blinded to sample identity evaluated the specific immunocytochemical labelling reaction. For each condition, between six and nine independent specimens were evaluated.

Statistics

The SPSS/ PC software package (SPSS, Chicago, IL, USA) version 15.0 was used for data collection, processing and statistical data analysis. Statistical analysis was performed using the non-parametrical Mann–Whitney U signed rank test for comparison of the means. $p \le 0.05$ was considered statistically significant.

Results

Estradiol

Estradiol acts as a natural ER ligand (ERa and ERb). HUVEC stimulated with 1, 10 and 100 pmol/ml and 1, 10, and 100 nmol/ml E2, respectively, showed no immunopositivity of ERa after cultivation for up to 72 h. In contrast, ERb immunolabelling was already seen after cultivation for up to 72 h and could be up-regulated by administration of increasing amounts of E2, as indicated in Figure 1.

Estrone

Estrone is known as one of the natural metabolites of E2. HUVEC stimulated with 0.1, 1, 10, 100, 500 and 1000 nmol/ml estrone showed significant up-regulation of ERb compared to unstimulated controls ($p = 0.043$ for 0.1, 1, 100 and 500 nmol/ml and $p = 0.042$ for 10 and 1000 nmol/ml, respectively, Figure 2). Stimulation with 0.1, 1 and 10 nmol/ml estrone led to ERb immunolabelling in 70–80% of all cells investigated, whereas stimulation with 100, 500 and 1000 nmol/ml estrone led to immunopositivity of ERb in over 90% of HUVEC.

Ethinylestradiol

Oral contraceptives contain ethinylestradiol (EE), the most widely used synthetic estrogen receptor activator. Up-regulation of ERb immunolabelling was seen after stimulation with 1, 10 and 100 nmol/ml EE (Figure 3). This up-regulation was significant when compared to unstimulated controls ($p = 0.043$). Expression of ERb was seen in 50–60% of HUVEC investigated and was not up-regulated by administration of higher EE concentrations.

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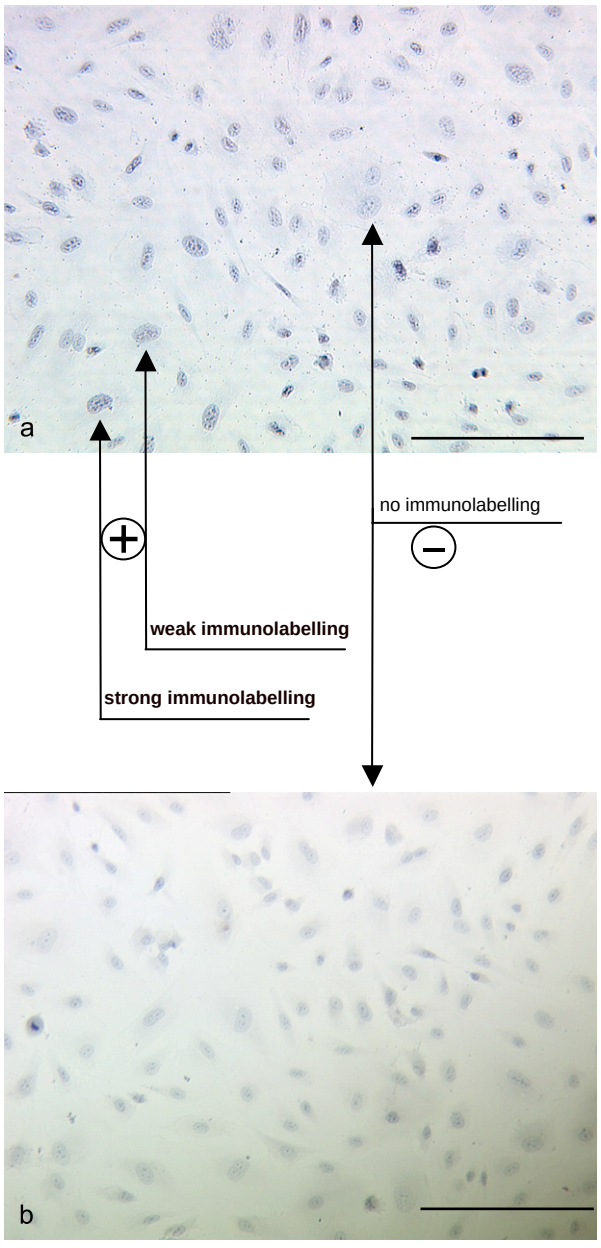

Figure 1. ERb in non-stimulated HUVEC cells (a). Arrows indicating nuclei with strong and weak immunolabelling (counted as positive) and nuclei without immunolabelling (counted as negative). (b) A representative negative immunolabelling for ERa. Scale bar = 200 mm.

Tamoxifen (SERM)

Tamoxifen is a widely used SERM, especially in treatment regimes of ER positive breast cancer patients. HUVEC stimulated with 0.1 and 1 nmol/ml Tamoxifen showed significant up-regulation of ERb compared to non-stimulated controls ($p = 0.043$), whereas addition of 10 and 100 nmol/ml Tamoxifen had no significant influence on ERb immunolabelling (Figure 4). In contrast, 500 and 680 nmol/ml Tamoxifen significantly reduced ERb immunolabelling in HUVEC ($p = 0.042$ and 0.043, respectively). Stimulation with 0.1 and 1 nmol/ml Tamoxifen lead to ERb immunolabelling in 35–45% of all cells investigated compared to 20–25% in non-stimulated controls. Higher concentrations of Tamoxifen (500 and 680 nmol/ml) reduced ERb immunopositivity to 5–10% of cells.

ICI 182.780

Blocking of the 17b-estradiol receptor can by achieved using ICI 182.780, a pure estrogen receptor antagonist. HUVEC stimulated with 0.1 and 1 nmol/ml ICI 182.780 showed significant up-regulation of ERb compared to non-stimulated controls ($p = 0.042$ and 0.043, respectively). Addition of 10 nmol/ml ICI 182.780 had no significant influence on the amount of ERb immunopositivity, whereas 100 and 500 nmol/ml ICI 182.780 significantly reduced ERb immunolabelling in HUVEC ($p = 0.043$ Figure 5). Stimulation with 0.1 and 1 nmol/ml ICI 182.780 lead to ERb immunolabelling in 35–70% of all cells investigated compared to 20–25% of controls. In contrast, administration of higher ICI 182.780 concentrations (100 and 500 nmol/ml) reduced ERb immunolabelling to 10–15%.

2,3-bis (4-hydroxyphenyl) proprionitrile (DPN) (pure estrogen receptor agonist)

HUVEC stimulated with 0.1, 1 and 10 nmol/ml DPN resulted in significant up-regulation of ERb compared to non-stimulated controls ($p = 0.042$, 0.043 and 0.043) (Figure 6). Addition of 100 and 500 nmol/ml DPN had no significant influence on ERb immunolabelling, whereas concentrations of 2000 nmol/ml DPN reduced significantly ERb immunolabelling in HUVEC ($p = 0.042$). HUVEC stimulated with 0.1, 1 and 10 nmol/ml DPN showed immunolocalisation of ERb in 35–50% of all cells investigated compared to 20–25% of controls. In contrast, high concentration of DPN (2000 nmol/ml) reduced ERb immunolabelling to nearly 15%.

Tumour necrosis factor alpha

HUVEC stimulated with 1, 10 and 100 nmol/ml TNFa showed significant up-regulation of ERb in all cases compared to non-stimulated controls ($p = 0.011$, 0.018 and 0.011, respectively) (Figure 7). After HUVEC stimulation with 1, 10 nmol/ml TNFa immunolabelling of ERb was seen in 80–90% of all cells investigated and was not

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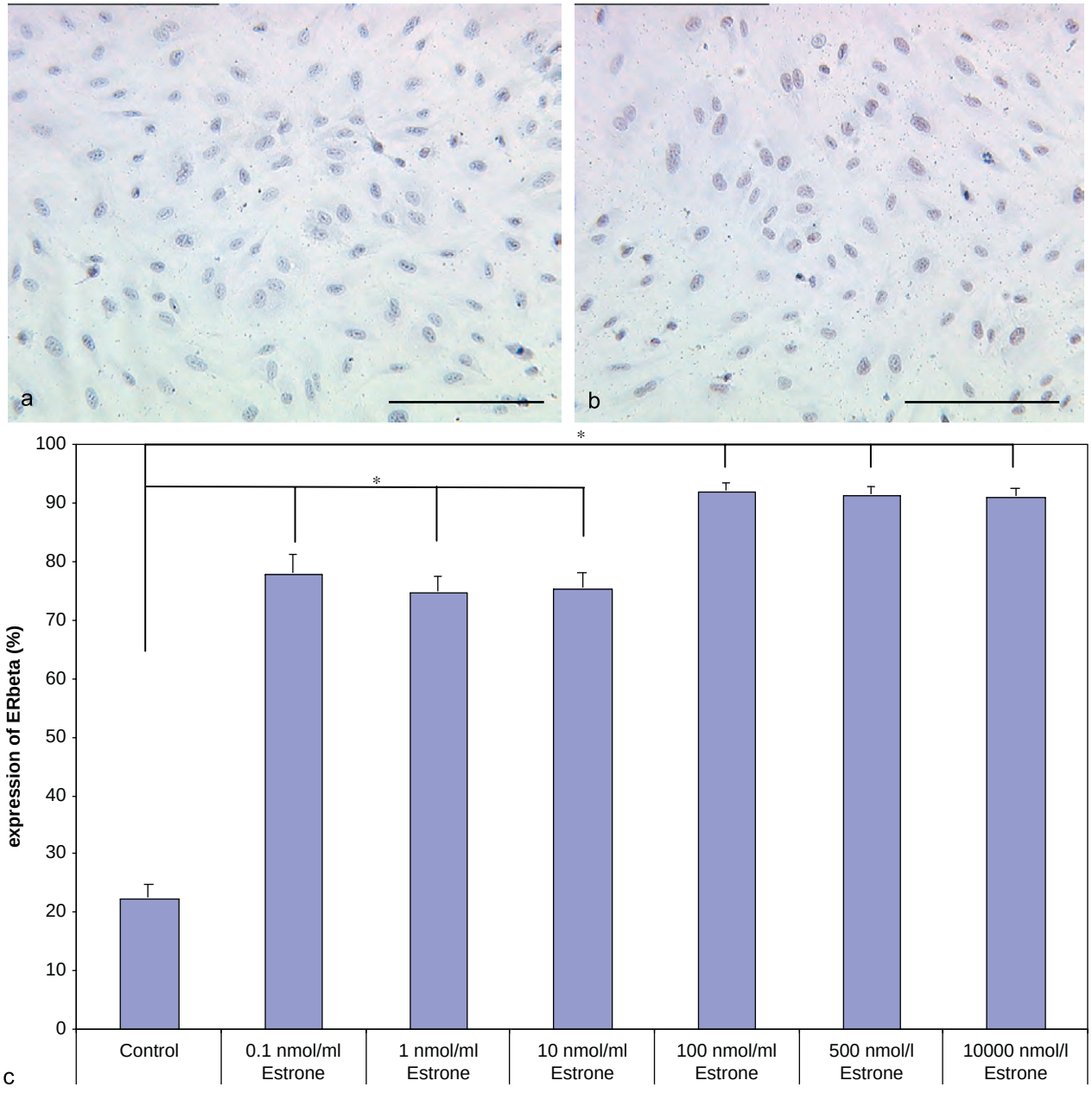

Figure 2. ERb immunopositivity is increased in HUVEC stimulated with 1 nmol/ml estrone (a) and 100 nmol/ml estrone (b). A summary of immunolabelling results is presented in (c). Scale bar = 200 mm.

further up-regulated by administration of 100 nmol/ml TNFa.

Discussion

In our study, we were able to demonstrate that estrogen derivatives, SERM as well as pure ER agonists or antagonists, act via ERb in HUVEC, whereas ERa seems not to be involved in this process. Metabolites of E2, as well as the synthetic estrogen EE, lead to an up-regulation of ERb protein immunolocalisation in a dose-dependent manner. Furthermore, SERM and ER antagonists act as ER activators when administered to HUVEC in low amounts, whereas high concentrations lead to ER down-regulation. With regard to ER agonists, administration of high concentrations lead to a down-regulation of ERb and only low concentrations activated ERb immunopositivity in HUVEC. The cellular effects of estrogens are mediated by binding to nuclear receptors (ER) that activate transcription of genes involved in cellular growth control. In a variety of cells ERa and ERb mediate

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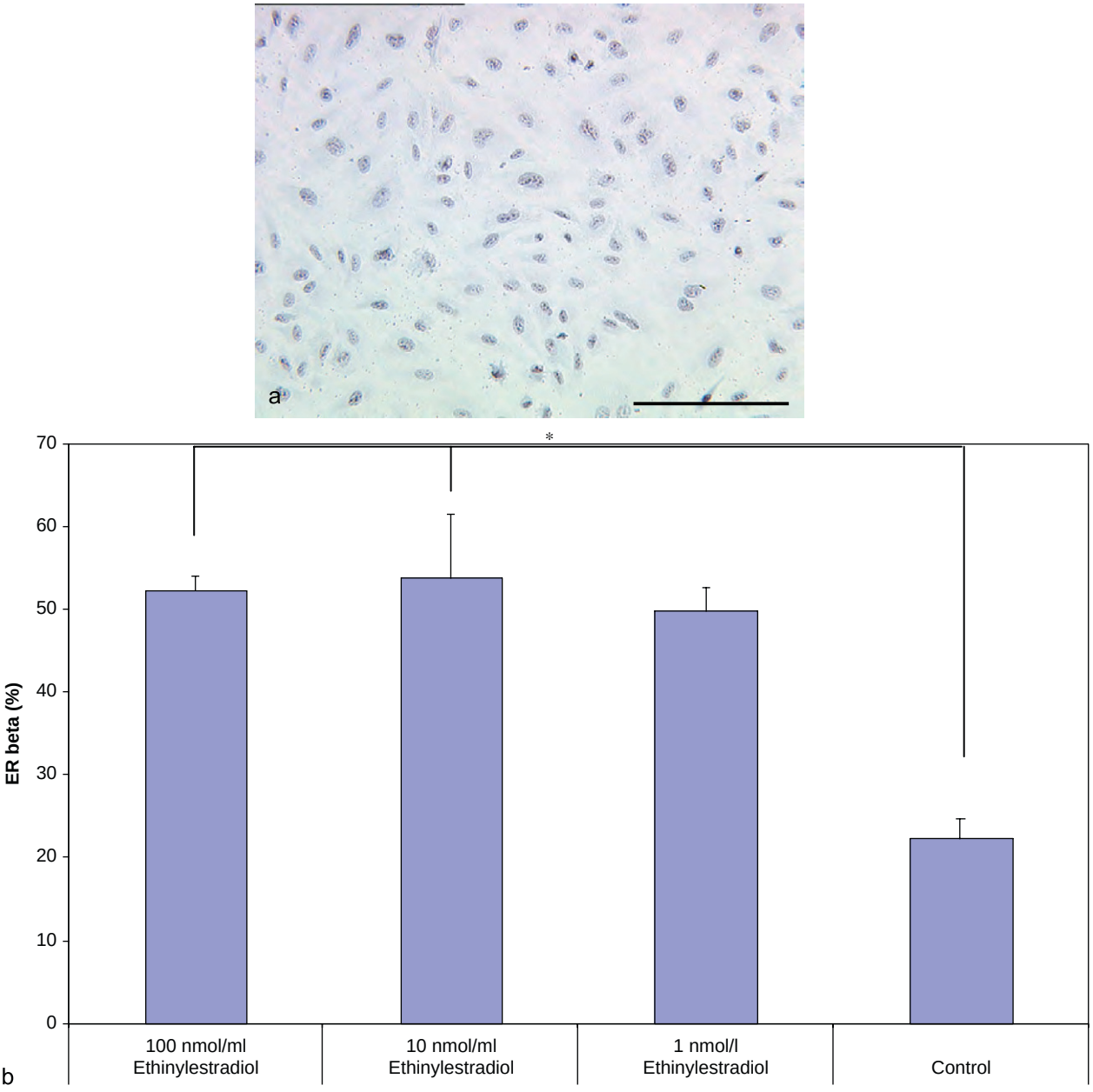

Figure 3. ERb immunopositivity is increased in HUVEC stimulated with 10 nmol/ml ethinylestradiol (a). A summary of immunolabelling results is presented in (b). Scale bar = 200 mm.

these effects in conjunction with a number of co-activators. Both receptors can directly interact with other members of the steroid receptor superfamily. A cross-talk exists between the estrogen-signalling pathways and the downstream signalling cascade initiated by growth factors, such as epidermal growth factor and insulin-like growth factors. Estrogens are also involved in the pathogenesis of a variety of neoplastic and non-neoplastic diseases, including breast cancer, endometrial cancer, endometriosis and uterine fibroids (Dhingra, 1999). In addition, estrogens are acquired risk factors for venous thromboembolism and endothelial cells play a major role in the pathogenesis of thrombophilia (Toth et al., 2007).

The concentration-dependent modulator effect of SERM in HUVEC could potentially arise from saturation of the ERb by co-regulators which trigger degradation or maintenance of a bound agonist for a period of time long enough to trigger removal and degradation (Jordan and O'Malley, 2007). Further studies are needed to investigate these potential mechanisms, but are beyond the scope of this paper.

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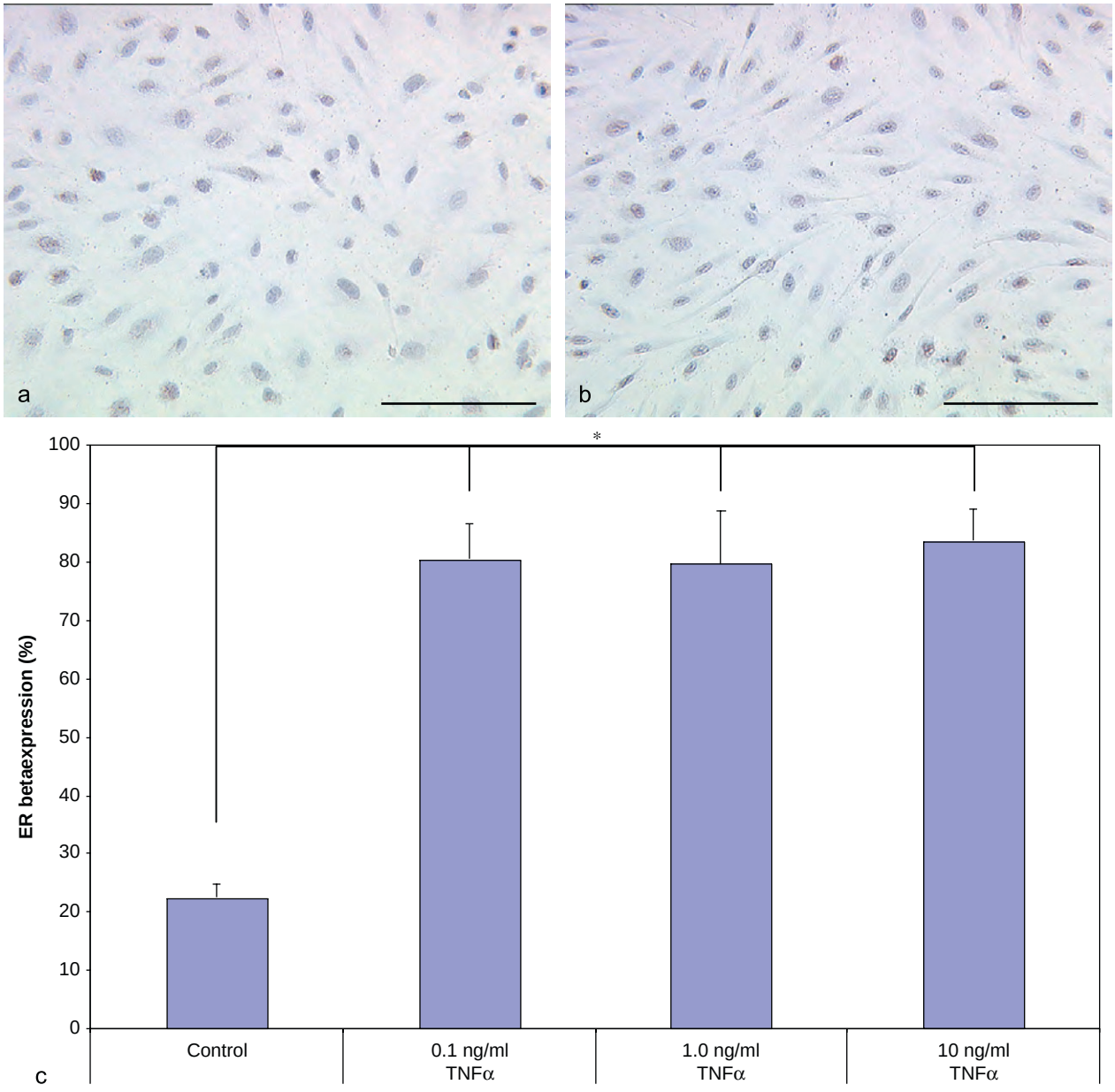

Figure 4. ERb immunopositivity is increased in HUVEC stimulated with 0.1 nmol/ml TNFa (a) and 10 nmol/ml TNFa (b). A summary of immunolabelling results is presented in (c). Scale bar = 200 mm.

Our results further support earlier investigations on ER expression in HUVEC. Ling et al. (2006) published data on the effects of 17b-estradiol on growth and apoptosis in HUVEC. They were able to demonstrate that E2 enhanced HUVEC growth in serum-enriched media in a concentration-dependent manner. The authors concluded that E2 effects are possible cellular mechanisms underlying female gender-associated cardiovascular protection. However, no differentiation between ERa and ERb expression was made. With regard to the effects of E2 and its metabolites on ERb immunolabelling in our study, we conclude that the known estrogenic effects in HUVEC were mediated by the non-classical ERb.

Nonetheless, conflicting results on ERa and ERb expression in HUVEC do exist (Jensen et al., 1998; Tatsumi et al., 2002; Herve et al., 2006). An ERa variant named ER46 was localized in the plasma membrane of HUVEC and it modulates membrane-initiated estrogen actions, including endothelial nitric oxide synthase (eNOS) activation (Li et al., 2003). Further studies on ER46 expression and activation are necessary to elucidate possible interactions between ERb and ER46.

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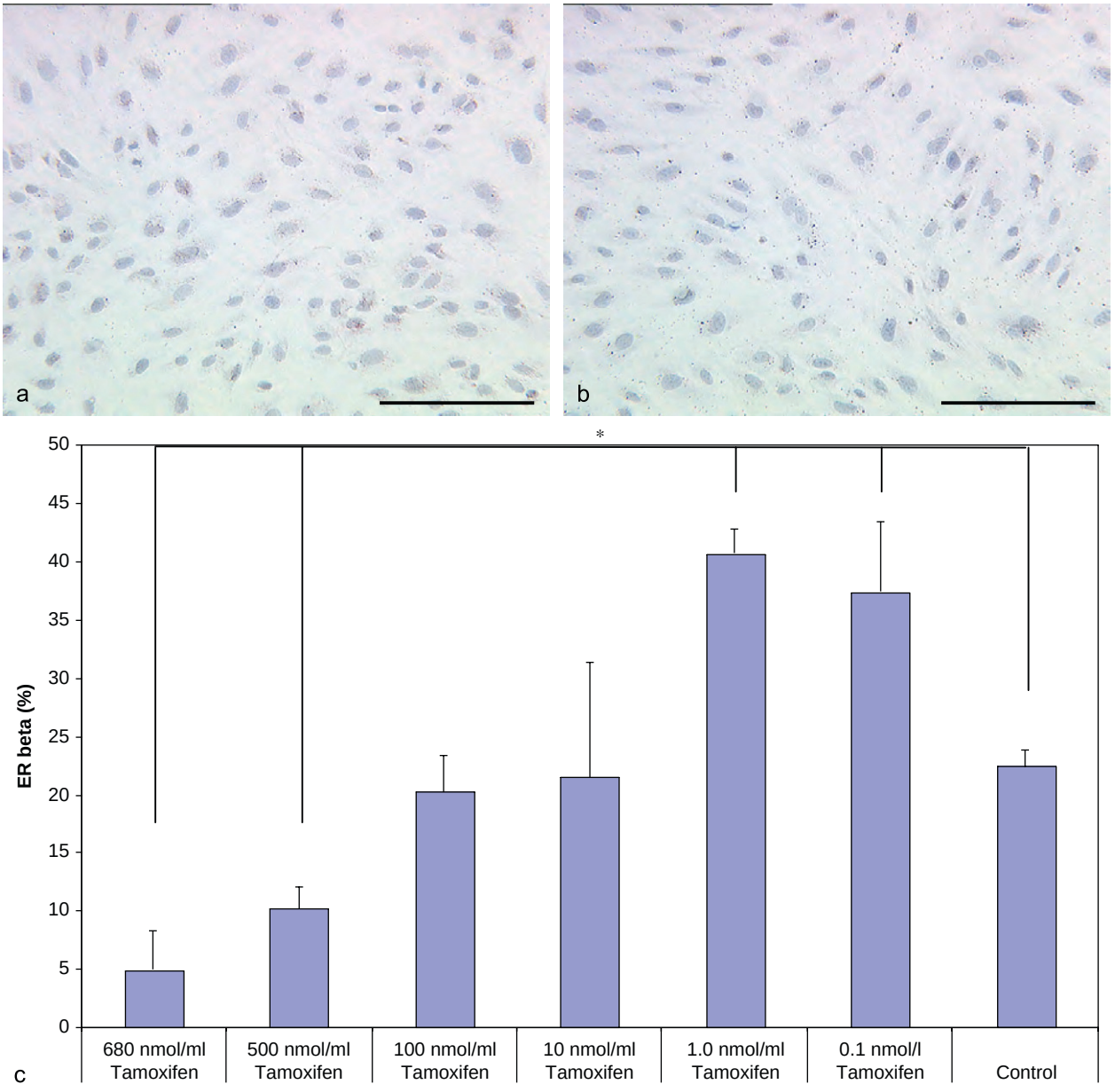

Figure 5. ERb immunopositivity is increased in HUVEC stimulated with 1 nmol/ml tamoxifen (a), whereas cells stimulated with 100 nmol/ml tamoxifen (b) showed no significant changes compared to non-stimulated controls. A summary of immunolabelling results is presented in (c). Scale bar = 200 mm.

So far, there are no systematic data on the effects of SERM and pure ER antagonists on ERb protein expression. We were able to show adverse effects depending on the administered concentration of SERM or pure ER antagonists. Both SERM and pure ER antagonists acted as ERb activators in low concentrations and as inhibitors in high concentrations. Lei et al. (2001) investigated the regulation of growth-regulated oncogene a (GROa) expression by E2 in HUVEC. Tamoxifen abrogated the down-regulation of GROa induced by E2. The authors concluded that GROa is regulated by ERa, as Tamoxifen abolished the E2 effect in more than 50% of cells. ERb expression was not investigated. Our results indicate that Tamoxifen regulates ER expression through ERb in HUVEC.

Besides SERM (like Tamoxifen), pure ER antagonists (i.e. ICI 182.780) were investigated to antagonize possible estrogenic effects on HUVEC (Hayashi et al., 1995; Florian et al., 2004; Oviedo et al., 2005). HUVEC incubated with five different concentrations of ICI 182.780 showed decreased cell viability and increased apoptosis (Soares et al., 2003). ICI 182.780 impaired angiogenesis by

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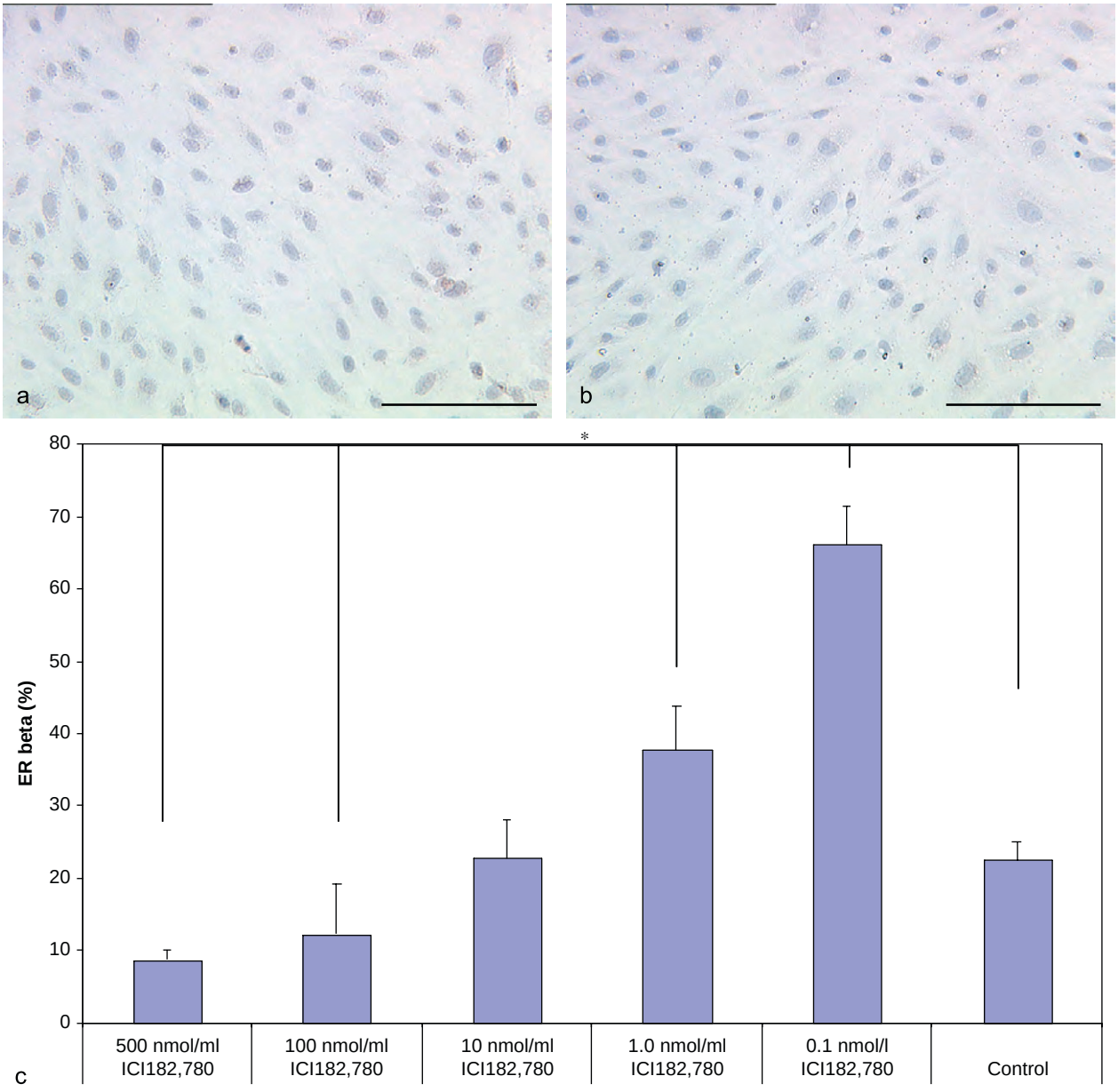

Figure 6. ERb immunopositivity is increased in HUVEC stimulated with 0.1 nmol/ml ICI 182.780 (a), whereas cells stimulated with 100 nmol/ml ICI 182.780 (b) showed significant reduced immunolabelling of ERb compared to non-stimulated controls. A summary of immunolabelling results is presented in (c). Scale bar = 200 mm.

preventing branching and capillary-like tubule formation and by activating apoptotic pathways in endothelial cells (Soares et al., 2003). We were able to demonstrate that ICI 182.780 acts as a pure ER activator in low concentrations, whereas its potential as ER antagonist is only present in high concentrations.

To our knowledge, there are no published data on the effects of the pure ERa and ERb agonist 2,3-bis (4-hydroxyphenyl) proprionitrile (DPN) on HUVEC. However, as expected, an up-regulation of ERb immunolabelling was seen at low concentrations, whereas administration of high concentrations leads to a down-regulation of ERb.

There are several investigations on the effects of TNFa on HUVEC (Miro et al., 2000; Silva et al., 2003; Ling et al., 2006; Pihusch et al., 2006; Szczepanski et al., 2007). However, as far as we are aware, no effects of TNFa administration on ERb expression have been published. We were able to demonstrate that TNFa leads to an activation of ERb expression up to 80–90% of investigated HUVEC, which could

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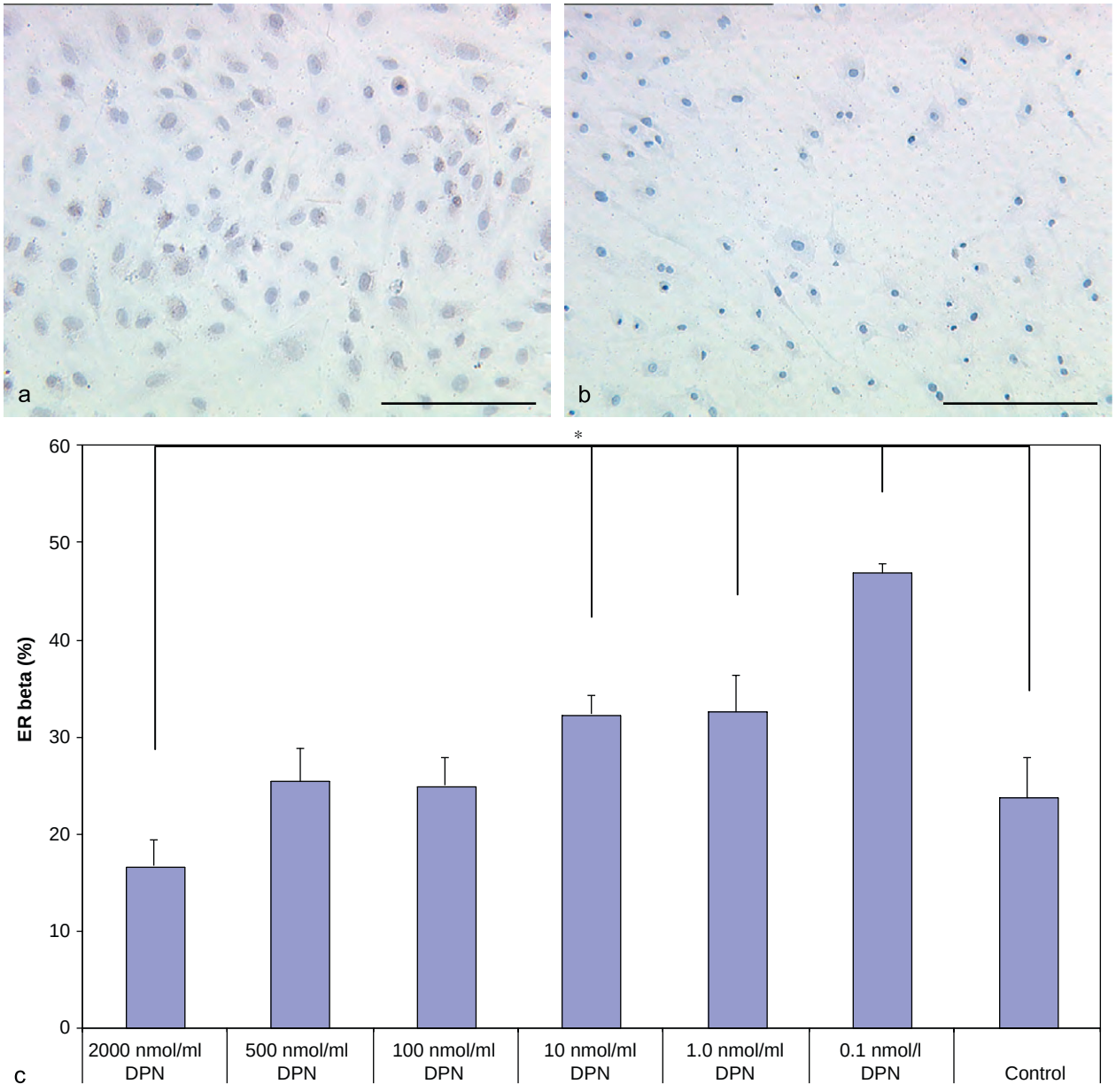

Figure 7. ERb immunopositivity in HUVEC stimulated with 0.1 nmol/ml DPN (a), whereas cells stimulated with 2000 nmol/ml DPN (b) showed significant reduced immunolabelling of ERb compared to non-stimulated controls, A summary of immunolabelling results is presented in (c). Scale bar = 200 mm.

not be further up-regulated by higher amounts of TNFa.

In summary, our study showed that effects of natural and synthetic female sex hormones as well as SERM, pure ER agonists and antagonists on HUVEC are mediated via the ERb pathway. Estrogen and its metabolites up-regulated ERb protein expression in a concentration-dependent manner, whereas pure ER agonists and antagonists exhibit adverse effects depending on the concentrations investigated.

Acknowledgments

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Glycodelin A Induces a Tolerogenic Phenotype in Monocyte-Derived Dendritic Cells In vitro

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Keywords
DC-SIGN, dendritic cells, feto-maternal interface, glycodelin, tolerance

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Problem
Successful mammalian pregnancy requires a delicate immunological balance at the feto-maternal interface that allows the semi-allogeneic fetus to grow, while protecting mother and child from environmental pathogens. As in other mucosal tissues, antigen-recognition and -handling by professional antigen-presenting cells such as dendritic cells (DC) determine the course of the subsequent immune response. DC at the feto-maternal interface help shape this immunological equilibrium. Endometrial tissue secretes high quantities of glycodelin A (GdA) during the so-called fertile window (i.e. the time of implantation of the blastocyst).

Method of study
We investigated the effect of GdA on monocyte-derived DC (moDC) regarding surface marker expression, endopinocytotic activity, cytokine profile as well as lymphoproliferative activity.

Results
Upon pretreatment with GdA and subsequent maturation with tumor necrosis factor-a and interleukin (IL)-1b, moDC displayed a reduced expression of costimulatory molecules, an unchanged major histocompatibility complex-II expression and persistence of DC-SIGN positive cells. GdA-pretreated moDC had a higher endopinocytotic activity, an increased IL-10 production and a dose-dependent reduction in lymphoproliferative activity. GdA incubation alone did not alter the immature phenotype.

Conclusion
Our results suggest a model in which the human endometrium secretes high quantities of GdA during implantation and thereby helps to shape the unique immunological interaction between mother and fetus via decidual DC.

Introduction

Viviparity poses an immunological paradox as fetal tissues express alloantigens encoded by paternal polymorphic major histocompatability complex (MHC)-genes. The growing fetus is not merely tolerated by the maternal immune system during the time of pregnancy, but there is growing evidence that the unique immunological equilibrium at the feto-maternal interface plays an integral part during placentation.[1] Many factors that influence this unique immunological relationship are known

today. There are accumulating data that dendritic cells (DC) play a central role within this immunological feto-maternal network.[2,3] There is also evidence that maternal T cells are aware of fetal alloantigens, but acquire a transient state of antigen-specific tolerance during pregnancy.[4] DC are professional antigen-presenting cells (APC) and central regulators of immunity.[5] They can initiate primary T-cell immune responses by antigen-uptake and processing in inflamed peripheral tissues followed by migration to T-cell rich areas of peripheral lymph nodes, where they prime T cells via antigen presentation and simultaneous costimulation. At the same time, there is evidence that DC also play a role in maintaining peripheral tolerance and act either as inducers or as suppressors of antigen-specific immunity.[6,7] The human decidua has long been known to be a tissue of antigen-presenting properties.[8] Only recently, however, a set of data is accumulating regarding the role of professional APC in the human placenta.[2,9,10] The capacity of DC to induce immunity or tolerance seems to be dependent on their state of activation and can be influenced by exogenous factors.[11] DC found at the feto-maternal interface have been shown to exhibit an immature or semi-mature phenotype with a high proportion of cells expressing the C-type lectin receptor DC-SIGN.[12] Glycodelin A (GdA), formerly known as placental protein 14, constitutes one of many factors known today that influence the immunological interaction between mother and fetus.[1] GdA, a member of the family of lipocalines, is a secreted glycoprotein found in variable concentrations in the human genital tract. One of the main tissues of GdA-secretion is the human endometrium. Endometrial secretion of GdA is strictly dependent on the rise of progesterone levels during the luteal phase of the menstrual cycle and during early pregnancy and is secreted in high quantities (more than 10^5 lg/L in amniotic fluid of early human pregnancy).[13] GdA is known to have direct immunomodulatory effects. In a seminal series of studies, it has been shown to suppress reactivity of phytohaemagglutinin-stimulated lymphocytes *in vitro*.[14–16] GdA has also been shown to inhibit directly NK and B-cell function *in vitro*.[17,18] But, only recently, scientific interest was drawn to the fact that GdA might not be merely damping immune responses by direct blocking of NK- and T-cell function, but rather skewing the immune response to allow the fetal semi-allograft to implant into the endometrial tissue. Its immunomodulatory effects on T cells are mediated by binding to CD45 in a carbohydrate-dependent manner.[19] In stimulated T cells, it preferentially inhibits expression of interferon (IFN)-c, as opposed to interleukin (IL)-5 and IL-4 by reduced expression of chemokine (C-X-X motif) receptor 3 (CXCR3) and reduced repression of the transcriptional factor GATA-3.[20] Further suppressive effects are known regarding NK- and B cells.[18,21] So far, no effects have been described regarding DC, although a glycodelin receptor has been postulated on monocytes.[22] Only recently, a proapoptotic gene cascade has been characterized in monocytic cell lines and primary human monocytes upon GdA stimulation.[23] On the basis of the hypothesis that GdA might influence DC present at the feto-maternal interface and therefore foster successful implantation of the human blastocyst, we investigated the effect of isolated human GdA on phenotype, endopinocytosis, cytokine-secretion and lymphoproliferative capacity on monocyte-derived DC (moDC).

Materials and methods

Cell culture

All cell cultures were maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2% heat inactivated human pooled AB serum (BioWhittaker, Walkersville, MD, USA), 2 mm l-glutamine (Life Technologies, Paisley, UK), 50 U/mL penicillin, and 50 mg/mL streptomycin (both from Sigma, Munich, Germany), hereafter referred to as complete medium. All cultures were maintained at 37°C, 5% CO_2 and 95% humidity.

Isolation and Culture of Human DC

Dendritic cells were generated from peripheral blood mononuclear cells (PBMC), as described elsewhere, with minor modifications.[24] In brief, PBMC were isolated from healthy voluntary donors by standard density-gradient centrifugation on Ficoll separating solution (Biochrom), washed three times and resuspended in complete medium. PBMC (5×10^6/mL) were allowed to adhere in 75-cm^2 culture flasks for 60 min. Non-adherent cells were removed by pipetting. After an overnight incubation, the initially adherent cells were transferred into 6-well plates (1.5×10^6 cells/2 mL) in fresh complete medium supplemented with 1000 IU/mL granulocyte–macrophage colony-stimulating factor (GM-CSF) and

500 IU/mL IL-4. A sample of generated moDC was assessed for purity (especially bystander lymphocytes) before further use.

Reagents

GM-CSF (Leukine®) was purchased from Immunex (Seattle, WA, USA), IL-4 from Promega (Madison, WI, USA), and tumor necrosis factor-a (TNF-a) from R&D Systems (Wiesbaden, Germany); IL-1b was obtained from Strathmann Biotech (Hannover, Germany).

Purification of GdA

Glycodelin A was purified from pooled human midtrimester amniotic fluid as previously described.[25] Briefly, amniotic fluid was loaded onto a diethylaminoethyl-sepharose column and fractionated on a 50–500 mm NH_4HCO_3) gradient followed by gel filtration and a second anion exchange chromatography step on Resource Q. Final purification was obtained by hydrophobic interaction chromatography on Octyl-Sepharose and Resource-Phe (all columns: GE Healthcare, Freiburg, Germany). All batches were tested for purity by Western blot (primary goat-antibody: sc-122291; Santa Cruz, Heidelberg, Germany) (Fig. 1). Biotinylated secondary anti-goat antibodies (PK-6105) and Avidin-Alkaline Phosphatase Complexes (AK-5000) were purchased at Vector Laboratories (Burlingame, CA, USA) and were used according to the manufacturer's instructions. Final tagging was performed using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (BCIP/NBT; Sigma) yielding purple staining of labeled bands.

Batches of purified GdA were tested for endotoxin contamination by its lack of TNF-a induction on PBMC. All samples were collected from patients who underwent reduction for polyhydramnia for medical reasons. The local ethical committee approved the obtaining and handling of human material.

mAbs and Flow Cytometry

Mouse anti-human monoclonal antibodies (mAbs) and the appropriate isotype controls were all obtained from Becton Dickinson (Heidelberg, Germany). Anti-HLA-DR, (immunoglobulin IgG2b; APC-conjugated); anti-CD86 (IgG1; phycoerythrin-conjugated); anti-CD83 [IgG1, fluorescein isothiocyanate (FITC)-conjugated]; anti-CD14 [IgG2b; peridinin chlorophyll protein (PerCP)-conjugated]. For FACS-analysis, 10^5 moDC suspended in 100 lL of phosphate-buffered saline (PBS) were incubated with 10 lL of the fluorochrome-labeled mAbs for 20 min on ice. After the staining procedure, the samples were washed once in PBS and measured immediately (FACS-Calibur; Becton Dickinson). Data were analysed using CellQuest Pro software (version 5.2.1; Becton Dickinson).

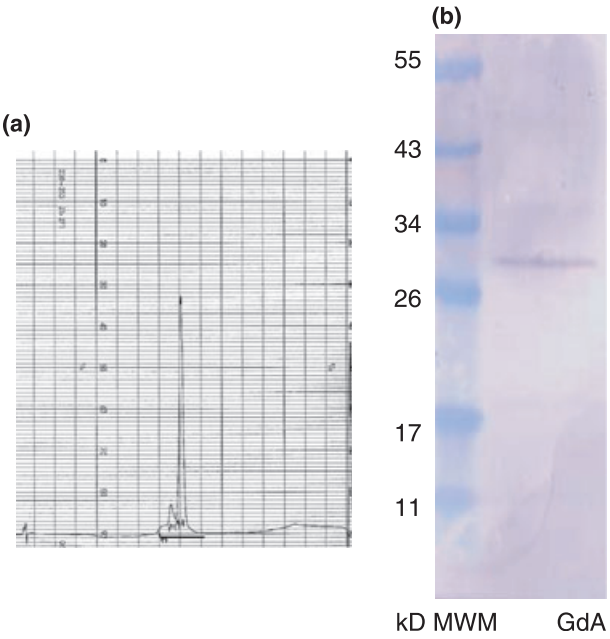

Fig. 1 High-performance liquid chromatography (HPLC) (a) and western blot analysis (b) of purified glycodelin A (GdA). First GdA was purified using a three-step HPLC isolation protocol described in Materials and methods. An example of the final isolation step is presented in (a). GdA fraction is marked with a bar. For protein identification and purity verification, a Western blot was performed (b) yielding an appropriate band at 28-kDa. MWM, molecular weight marker.

Endopinocytotic Activity

Cells were washed and endopinocytotic activity was assessed adding FITC-dextran (0.5 mg/mL) (M_r 40,000) to the culture medium for 30 min at 37°C (control on ice), as described elsewhere.[26] In brief, immature, untreated DC served as positive controls when incubated at 37°C. Incubation on ice, on the other hand, abrogates endopinocytosis and was therefore used as a negative control. DC that had been matured in the presence or absence of 25 lg/mL GdA or with GdA alone were tested for their ability to take up FITC-dextran. After 30 min, cells were washed

three times to eliminate all FITC-dextran attached to the outer cell membrane and subsequently incorporated FITC-dextran was analysed by flow cytometry.

Lymphoproliferative Activity

Dendritic cells were harvested and cocultured in complete medium with a constant number of allogeneic non-adherent PBMC (2×10^5/200 lL) in 96-well round-bottom microtiter plates at ratios ranging from 1:10 to 1:160. DNA-synthesis was measured using the Cell Proliferation enzyme-linked immunosorbent assay (ELISA), 5-bromo-2-deoxyuridine (BrdU; chemiluminescent) (Roche Diagnostics, Mannheim, Germany). In short, cells were labeled by the addition of BrdU for 12 hr. BrdU is incorporated in place of thymidine into the DNA of proliferating cells. Cells were dried, fixed and the DNA denatured. Subsequently anti-BrdU, peroxidase-labeled antibody was added binding to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes were detected by a subsequent substrate reaction. The reaction product was quantified by measuring the light emission on a luminescence ELISA reader (MRX; Dynatech Lab, Burlington, MA, USA) at 450 nm wavelength.

Cytokine Aecretion

Cytokine secretion was analyzed by ELISA. BD OptEIA™ Human IL-10 and Human IL-12 (p40) ELISA set were purchased from Becton Dickinson and applied according to the manufacturer's instructions. Supernatants of cell cultures were collected 48 hr after stimulation and analysed.

Statistical Analysis

Data are expressed as median ± S.E.M. Statistical significance was determined by the Student's t-test for paired samples of original values. Differences were considered statistically significant for $P < 0.05$.

Results

Pre-incubation of moDC with GdA Results in Reduced Maturability

Immature moDC were characterized by the absence of CD14, CD83, CD86 and intermediate expression of MHC class II as well as DC-SIGN. To test the influence of GdA on their maturation processes, moDC were incubated with 0, 10, 25 or 50 lg/mL GdA over the course of 24 hr, washed extensively and subsequently matured using a standard combination of TNF-a (1000 U/mL) and IL-1b (10 ng/mL).[11] After 48 hr, the morphological changes in the maturation process were assessed by flow cytometry. moDC that were exposed to GdA showed a diminished expression of CD83 and CD86 compared to matured DC (Fig. 2). In all tested conditions, moDC stayed negative for CD14 (data not shown). Incubation with GdA alone had no significant effect on the expression of costimulatory molecules. Upon maturation, untreated DC showed the expected increase in costimulatory- and MHC-molecules as well as a reduced expression of DC-SIGN. However, moDC that were subject to pretreatment with GdA only marginally increased the expression of costimulatory and MHC-II molecules compared to their unstimulated counterparts. As for DC-SIGN, we detected a mean of fluorescence intensity that lay in between matured and immature moDC. In four of six independent experiments performed, a distinct DC-SIGN positive population was seen (Fig. 2).

A similar pattern was seen for 50 lg/mL GdA (data not shown), whereas pre-incubation with 5 lg/mL GdA had only a marginal effect on the surface markers tested. However, no distinct DC-SIGN positive moDC populations were detected when pre-incubation was performed with low concentrations of GdA (data not shown). In all cases, viability of the cells was assessed using the trypan blue exclusion method and was comparable between the different conditions and time points. At higher concentrations (starting at 100 lg/mL), we encountered increasing cytotoxic effects as confirmed by trypan blue exclusion (data not shown).

Pre-incubation with GdA Leads to Diminished Endopinocytotic Activity

A functional parameter of immature moDC is their ability to constantly take up antigen and present it via MHC molecules without concurrent costimulation. Upon maturation, however, moDC downregulate endopinocytosis and present their antigen-content together with costimulatory molecules in a stable fashion leading toward immunostimulation.[27] To test the influence of GdA-pretreatment on endopinocytotic activity, moDC were incubated with FITC-dextran for 30 min and uptake of FITC-dextran was measured

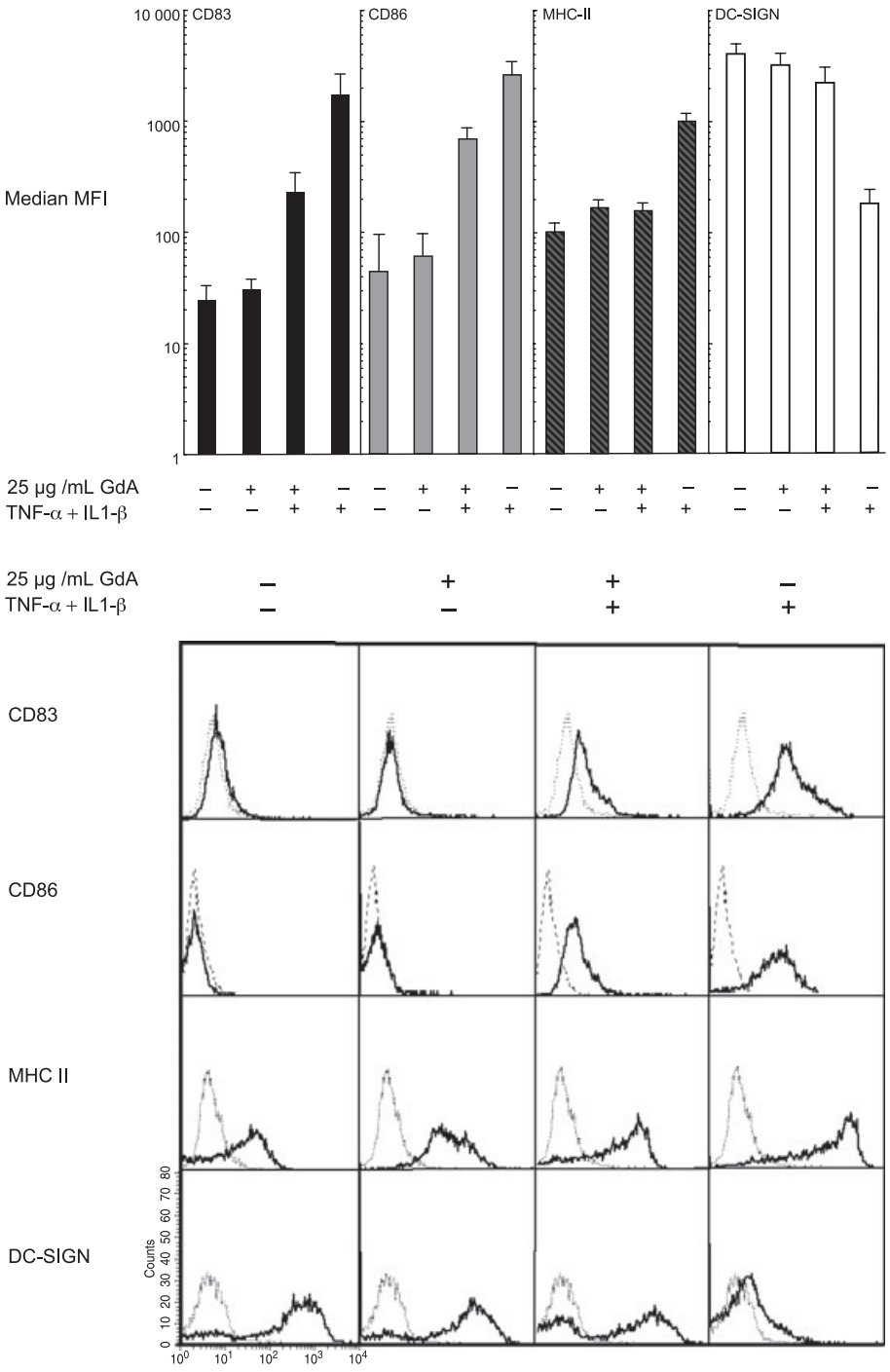

Fig. 2 Diminished expression of maturation markers and persistence of DC-SIGN after pretreatment with 25 lg/mL glycodelin A (GdA). Monocyte-derived DC were pretreated with 25 lg/mL GdA for 24 hr and subsequently matured with tumor necrosis factor-a (1000 lU/mL) and interleukin-lb (10 ng/mL) and were subsequently analysed by flow cytometry after 48 hr, as described in Materials and Methods. One representative experiment out of six is depicted in the lower segment. Dotted lines indicate isotype-controls. In the upper part, the median mean fluorescence index of all six experiments is shown accompanied by the standard error of mean (±S.E.M.). Conditions are marked by ± respectively.

after 24 hr by flow cytometry. Pretreatment with GdA resulted in persistence of endopinocytotic activity regardless of external maturation stimuli. A significant reduction in the capacity to downregulate endopinocytosis was detected when comparing matured and GdA-pretreated moDC (Fig. 3).

Preincubation with GdA Dose Dependently Reduces Lymphoproliferative Activity of moDC

To determine further the functional properties of GdA-pretreated and subsequently activated moDC, we assessed their capacity to stimulate allogeneic-mixed lymphocytes. DC–T cell ratio was set at 1:10–1:160. (1:10, 1:40, 1:80, 1:160). At concentrations of 10 lg/mL GdA, lymphoproliferative capacity upon maturation of moDC was only slightly diminished compared to matured moDC. At a concentration of 25 lg/mL GdA, subsequently matured moDC showed a marked reduction in their capacity to proliferate allogenic lymphocytes (Fig. 4). Compared to unstimulated moDC, GdA alone – without subsequent maturation – induced an approximately two-fold increase in lymphocytic proliferation. These proliferation rates were comparable to those seen with subsequent maturation. An increase in GdA concentration (25 and 50 lg/mL) did not alter the proliferative behaviour (data not shown).

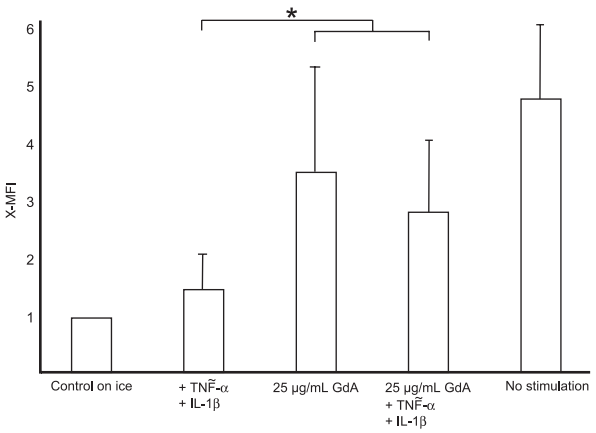

Fig. 3 Preincubation with 25 lg/mL glycodelin A (GdA) leads toward persistent endopinocytotic activity regardless of external maturation stimuli. Dendritic cells were incubated with fluorescein isothiocyanate-dextran at 37°C for 30 min. Mean data of five experiments are shown. Data are normalized against controls on ice and shown as multiples of the mean fluorescence index (x-MFI). Error bars indicate standard error of mean (S.E.M.). Bracket and asterisk indicate statistical significance.

GdA Pretreament Results in an Increased Secretion of IL-10 by moDC While Decreasing IL-12 Secretion

Upon activation, moDC are known to produce IL-12 as well as IL-10 on various stimuli and seem to characterize their immunogenic behaviour.[28,29] TNF-a and IL-1b stimulated moDC showed a median IL-12 p40 secretion of 4256 pg/mL (range: 6859–2615 pg/mL) and a median IL-10 secretion of 83 pg/mL (range: 165–23 pg/mL). This pattern changed toward an IL-10-dominant cytokine milieu once GdA was added before maturation. In this case, a median IL-12 p40 secretion of 1079 pg/mL (range: 2361–108 pg/mL) was noted, while IL-10 was detected in a median concentration of 311 pg/mL (range: 475–197 pg/mL). Low concentrations of IL-10 were detected in immature moDC [42 pg/mL (range: 23–55 pg/mL)] and GdA-treated moDC without subsequent maturation [69 pg/mL (range: 80–50 pg/mL)] with only trace IL-12 p40 detectable in these conditions (Fig. 5). High concentrations of GdA (50 lg/mL) did not change this cytokine pattern, whereas this effect was not statistically significant at concentrations of 5 and 10 lg/mL (data not shown).

Discussion

In this article, we are presenting data on the influence of GdA pre-incubation on moDC. This in vitro model is based on the assumption that ex vivo generated moDC may exhibit similar physiological behavior toward human decidual DC, which are predominantly of myeloid origin.[10] We show that moDC alter their immunological phenotype upon pretreatment with GdA.

Isolation of GdA

Glycodelin A was isolated and purified from pooled amniotic fluid by fast protein liquid chromatography and afterwards checked for purity and for lipopoysaccharide (LPS) contamination. High-performance liquid chromatography analyses of the purified product revealed one main peak and a small secondary peak in its immediate proximity. This small peak belonging to the same protein is known to be caused by changes in glycosilation e.g. desialization of the same purified protein.

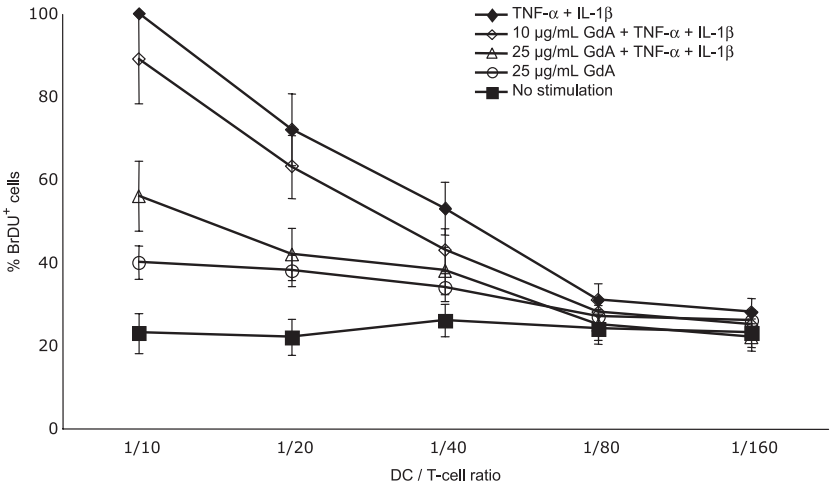

Fig. 4 Pretreatment with glycodelin A (GdA) leads toward a dose-dependent reduction in lymphoproliferative activity in spite of subsequent maturation. Monocyte-derived dendritic cells (moDC) were pretreated with the indicated concentrations of GdA for 24 hr and subsequently matured with tumor necrosis factor-a (1000 IU/mL) and interleukin-Ib (10 ng/mL). moDC were harvested and cocultured with allogenic non-adherent peripheral blood mononuclear cells in 96-well microtiter plates at the indicated ratios. 10 lM 5-bromo-2-deoxyuridine was added and its incorporation was determined after 72 hr of coculture. The data shown represent six independent experiments with the error bars indicating standard error of mean (±S.E.M.). Data were normalized against conditions in which untreated and matured DC were used in a DC/T-cell ratio of 1:10.

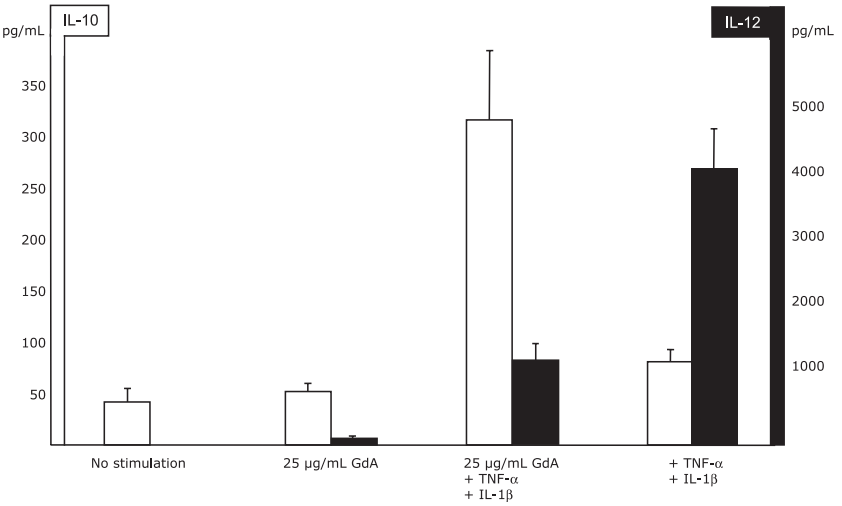

Fig. 5 Interleukin (IL)-10 dominant pattern of matured glycodelin A (GdA)-pretreated monocyte-derived dendritic cells (moDC). moDC that are pretreated with 25 lg/mL GdA before maturation with tumor necrosis factor-a (TNF-a; 1000 IU/mL) and IL-Ib (10 ng/mL) exhibit a IL-10^{high} IL-12^{low} cytokine pattern, whereas incubation with GdA without subsequent maturation does not lead toward an induction of IL-10. Maturation of DC with TNF-a and IL-Ib alone resulted in an IL-10^{low} IL-12^{high} cytokine pattern. Mean data of six independent experiments are shown. Error bars indicate standard error of mean (S.E.M.). Bracket and asterisk indicate statistical significance.

Surface Marker Expression

In our in vitro model, maturation of moDC with TNF-a and IL-1b led toward a fully matured phenotype with high expression of costimulatory molecules (CD83, CD86), MHC-II molecules and downregulation of CD 209 (DC-SIGN). Incubating moDC with 25 lg/mL GdA for 24 hr had no influence on the expression of these surface markers. Preincubating moDC with GdA, however, changed

the way moDC reacted on maturation stimuli. Compared to standard maturation with TNF-a and IL-1b alone, we saw a markedly reduced expression of CD80 and CD83, pointing toward a reduced T-cell stimulatory capacity. We also detected an intermediate level of expression for MHC-II molecules upon pretreatment with GdA. This is consistent with the notion that semi-matured DC may be tolerogenic under steady-state conditions.[30] Fitting into this picture was a persistence of DC-SIGN as a marker of immature moDC. Interestingly, we saw a distinct DC-SIGN positive population on GdA-pretreated and subsequently matured moDC in four of six independent experiments.

Interaction Between GdA and DC-SIGN

Circumstantial evidence points toward an interaction between DC-SIGN and GdA. DC-SIGN is a type II C-type lectin that binds with high affinity distinct carbohydrate structures, such as glycoconjugates, with a branch of at least three mannose structures (high mannose structures) as well as fucosylated Lewis blood group antigens (e.g. Lewis^x).[31,32] van Liempt et al.[33] reported that fucosylated Lac-di-NAc-N-glycans exhibit the highest binding efficiencies to DC-SIGN compared to a variety of other carbohydrate structures. GdA is known to carry all of these binding partners for DC-SIGN. Apart from high mannose structures, glycans found on GdA are blood group Lewis^x and the Lac-di-NAc analog of Lewis^x.[34] GdA is the only known mammalian glycoprotein carrying fucosylated Lac-di-NAc-N-glycans, which happen to be the strongest characterized binding partner for DC-SIGN and are otherwise only found on helminths like *Schistosoma mansonii*.[35] Furthermore, functional data support DC-SIGN mediated actions of GdA. In a recent report, Nonaka et al.[36] present repression of LPS-induced maturation of moDC that was dependent on interaction between DC-SIGN and Lewis blood group antigens, similar to those found on GdA. Subsets of myeloid DC are known to express DC-SIGN upon different stimuli including IL-10.[37] It is therefore tempting to speculate that GdA induces an antigen-dependent expression of DC-SIGN within a subset of moDC. There is an intriguing similarity between the effects we see when incubating moDC with GdA to other experiments looking into the behavior of DC toward *Schistosoma egg antigen* (SEA) that expresses GdA-typical Lac-di-NAc-N-glycans.[38] In these experiments, the authors show that SEA inhibits poly-I:C or LPS-induced DC activation and cytokine production and down-modulates T helper (Th)1 responses of phorbol 12-myristate 13-acetate-stimulated naïve T cells as detected with intracellular IL-4 (Th2) and IFN-c (Th1) flow cytometric staining. Similar to our experiments, the authors see a reduction in IL-12 p40 secretion in LPS or poly I:C stimulated DC upon simultaneous incubation with SEA, while IL-10 secretion remains high with low concentrations of SEA. In addition, the authors were able to show dependence of these effects on interaction with DC-SIGN. Although there are considerable differences in the experimental setup (e.g. 24 hr GdA-preincubation versus simultaneous SEA-incubation), there seems to be a similar immunological reaction on DC. In our experiments, we do not prove binding or signaling via DC-SIGN because of scarcity of purified human GdA. Recombinant glycodelin, on the other hand, exhibits a different glycosilation pattern and may therefore show different binding capacity and biological actions.

Cytokine Secretion

One of the decisive factors discriminating between immunostimulatory and tolerogenic DC is their cytokine secretion. moDC have been shown to secrete IL-12, as one of the pivotal Th1 cytokines, upon various maturation stimuli.[39,40] We were able to show in previous experiments that moDC secreted IL-12 upon maturation with TNF-a, prostaglandin E_2 and purine derivatives.[26] Other groups have identified cytokine cocktails that induced stabile maturation in moDC.[41] There are data supporting the notion that IL-12 secretion strictly depends on Toll-like receptor (TLR)- or CD40-ligation.[42] In our experiments, autologous bystander T cells (5–7%) that could not be eliminated during the adherence-sorting process provided CD40 ligation. IL-10, the hallmark cytokine involved in Th2 responses, has been shown to be produced by DC upon exposure to respiratory antigens, for example.[6] Another possible source of IL-10 are (regulatory) T cells, as bystander T cells were present in our moDC cultures. GdA is known to suppress directly T-cell reactivity and to induce Th2 cytokines like IL-4 or IL-13. IL-10, however, was not measured in these experiments.[20,43] Given the purity of the DC preparation, the most probable source of IL-10 upon pretreatment with GdA in our experiments seems to be moDC. There is conflicting

evidence on the induction of apoptosis in monocytes by GdA.[23,43] The secretion of IL-10, we saw in our experiments, however, argues against a direct cytopathic effect of GdA on moDC. Moreover, we controlled viability by trypan blue exclusion leading toward comparable results at the GdA-concentrations used. At higher concentrations, however, we also came across cytopathic effects, although we did not distinguish between apoptosis and necrosis. In a recent publication on this issue, it was observed that TNF-a stimulation of monocytes leads toward an activation of NF-jB, rescuing monocytes from apoptosis induced by recombinant GdA.[23] In these experiments, TNF-a-induced NF-jB activation was significantly reduced upon incubation with recombinant GdA. As IL-12 is expressed under an NF-jB promotor, this fact may shed some light on intracellular signaling of IL-12 secretion upon GdA-pretreatment. A similar cytokine pattern, as we have seen upon GdA preincubation, has been reported for mononuclear cells isolated from human deciduas.[44] Isolated ($CD45^{+}$; $CD3^{)}$; $CD14^{)}$; $CD16^{)}$; $CD19^{)}$; $CD20^{)}$; $CD56^{)}$; HLA-DR^{bright}) DC from early human pregnancy were shown to secrete less IL-12 upon LPS-activation than their counterparts isolated from peripheral blood.[45] Spontaneous secretion of Th2 cytokines including IL-10 has been reported for mononuclear cells during the course of pregnancy.[46,47] IL-10 is associated in mice and humans in the mechanisms of maternal tolerance of normal pregnancy.[48,49] IL-10-deficient mice, however, are fertile, but show an altered growth trajectory *in utero* and after birth.[50] Taken together, the morphological features regarding surface marker expression as well as cytokine expression point toward a tolerogenic state of moDC that were exposed to GdA before activation. Two further assays added functional data to this picture. Immature moDC are known to exhibit a high degree of endopinocytotic activity that is lost upon maturation leading toward an immunostimulatory phenotype.[51] Immature DC, however, transiently present incorporated antigen via MHC molecules on their cell surface, yet do not express costimulatory molecules. There is published data that semi-mature DC, that have received maturation signals yet fail to downregulate endopinocytosis while upregulating costimulatory molecules, exhibit tolerogenic properties.[11] When pretreating moDC with GdA at least part of this endopinocytotic activity is retained, again indicating toward a semi-mature state and regulatory capacities. Furthermore, GdA-pretreatment led toward a dose-dependent decrease in T-cell stimulatory activity, the hallmark of immunogenic DC. This finding is consistent with the finding that isolated early pregnancy decidual myeloid DC also showed a reduced T-cell stimulatory capacity.[3] Our experimental setup does not allow proving an antigen-specific tolerogenic behavior and may not even be part of the physiological role of decidual DC. Our data, however, do provide evidence that DC lose their immunogenic, T-cell stimulatory capacity upon encounter of GdA that is part of the intricate network of the feto-maternal interface.

Our data point toward a putative model in which GdA that is endometrially secreted in high quantities during the first phase of pregnancy helps shape the unique immunological interaction between mother and fetus via DC-SIGN expressed on decidual DC. This mechanism is mimicked by the unique carbohydrate structure present on helminths like *S. manonii*.

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Conflict of interests

The authors state that there are no conflicting financial or commercial interests.

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Flowcytometric assessment of fetomaternal hemorrhage during external cephalic version at term

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Abstract

External cephalic version (ECV) at term is a safe procedure and reduces the incidence of cesarean sections for breech presentation. One of the known complications, however, is an ECV-related disruption of the placental barrier and a subsequent transfusion of fetal blood into maternal circulation. While the incidence of ECV-related fetomaternal hemorrhage (FMH) has been determined recently in a large trial using a manual Kleihauer-Betke test (KBT), questions remain on the amount of ECV-related FMH. KBT, which detects fetal red blood cells (RBC) on the basis of acidic resistance of fetal hemoglobin (HbF), is known to be a sensitive test, yet prone to procedural errors limiting its accuracy in quantifying FMH. In this study we investigated 50 patients for FMH before and after ECV, using a dual-color flow cytometric test kit with a lower limit of quantification of 0.05% fetal RBC in maternal peripheral blood. Three patients had a quantifiable increase of fetal RBC detected after ECV (0.06%; 0.08%; 0.1%). None of these subtle increments was predictable by ECV-related clinical parameters or translated into fetal compromise. Using a sensitive and accurate flow cytometric test method, our data provide further assurance to mothers on the safety of ECV at term.

Keywords: Carbonic anhydrase; external cephalic version; F-cells; fetomaternal hemorrhage; flow cytometry.

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Introduction

Planned cesarean sections have become the standard mode of delivery for women with breech presentation at term. To reduce the number of cesarean sections for this indication, current evidence recommends external cephalic version (ECV) as a safe procedure, which improves the chance of cephalic birth given that the appropriate selection criteria and safety measures are applied [11]. Although there are solid data on its safety, specific complications of ECV have been reported [5]. Most notable among these complications is the disruption of the placental circulation-barrier, with ensuing fetomaternal hemorrhage (FMH), commonly defined as a transfusion of an equivalent of more than 30 mL fetal blood into the maternal circulation. However, none of the small studies or case reports addressing this issue had the necessary statistical power to provide reliable data on the incidence of FMH. Only recently has a cohort study encompassing a total of 1311 women been published; this work tested the amount of fetal cells in the maternal circulation before and after ECV using a manual Kleihauer-Betke test (KBT) [2]. In this well-powered trial, 3% of all patients had a positive KBT after ECV, with one patient having an equivalent of more than 30 mL fetal blood detectable in the maternal circulation. While providing solid data on the incidence of FMH in ECV, the authors stress that the known limits of the KBT are of concern [2, 17]. KBT, which differentiates fetal from maternal red blood cells (RBC) based on the relative resistance of RBCs containing hemoglobin F (HbF) to acid elution, is inexpensive, but known to be prone to a variety of procedural errors, making the quantification of FMH difficult [8]. Flow cytometric test methods have been evolving rapidly, and, by adding additional parameters to HbF, are able to control for HbF positive maternal RBC (s F-cells) that are present in women with sickle cell disease and thalassemia and in up to 25% of healthy women in the second trimester of pregnancy [14, 17]. Recently, Porra et al. evaluated a dual-color flow cytometric test method that identifies F-cells by additionally staining carbonic anhydrase (CA) [18]. As an inducible enzyme in the respiratory chain, CA is only present in adult RBCs, allowing for the specific quantification of more than 0.05% fetal RBC in maternal blood when analyzing $2 = 10^6$ RBCs. In the current study, we quantified the number of fetal cells before and after ECV using this dual-color flow cytometry test kit and correlated the amount of FMH with ECV-related parameters.

Materials and methods

A total of 50 women were included in this prospective observational study of all singleton breech pregnancies during the year 2007 that were subject to ECV in the department of obstetrics and gynecology Klinikum – Innenstadt of the Ludwig-Maximilians University in Munich. All women gave their informed consent, and the institutional Review Board approved the protocol. All women with breech presentation at term were evaluated with a scoring system before ECV. We integrated data regarding amniotic fluid index, fetal and placental position, enlacement of the umbilical cord, estimated fetal weight, parity, uterine tone and breech mobility (Figure 1). ECV was attempted among women with a score of) 8 points and was performed according to the current German guideline on delivery in breech presentation [10]. During an initial 30-min non-stress test, an i.v. line was inserted and 5 mL of total venous blood were collected into ethylenediaminetetraacetate (EDTA) anticoagulant. ECV was performed by an experienced consultant and attempted no more than three times. After a post-ECV CTG of 30 min we collected five additional mL of peripheral venous blood and labeled them as "post ECV".

The procedure was documented with special reference to presumed risk factors for FMH: pressure on placenta, use of tocolytic agent, failure of ECV, number of attempts, CTG decelerations and perceived pain (Figure 1).

Detection and quantification of fetal erythrocytes was performed with the Fetal Cell Count Kit II (IQ-Products, Groningen, The Netherlands). In brief, RBCs were washed three times in phosphate-buffered saline (PAA-Laboratories, Pasching, Austria) for 30 min in a formaldehyde-containing solution, washed once more, and then permeabilized with sodium dodecyl sulfate solution for 4 min at room temperature. RBCs were then stained with a fluorescein isothiocyanate-labeled monoclonal mouse anti-human fetal hemoglobin (HbF) antibody (recognizing the HbF alpha-chain) and a phycoerythin-labeled polyclonal rabbit anti-human CA antibody (recognizing the CA-II isoform). The additional antibody for CA removed the need to determine HbF threshold values, as it distinguished between the physiological maternal F-cells (HbF^{q}/CA^{q}) and the genuine fetal RBCs (HbF^{q}/CA^{-}). Prior to flow cytometry, optimal detector amplifications and compensations were determined using unstained or single-stained controls as well as cord-blood samples. Data acquisition was performed on a FACSCalibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). For quality control, spiking experiments were performed using a mixture of peripheral blood of nulliparous non-pregnant individuals and cord blood at various dilutions. Concomitant test controls to validate and monitor the quality of flow cytometric procedures were performed using FetalTrol® test samples (IQ-Products, Groningen, The Netherlands). In concomitant test controls and spiking experiments, we were able to confirm the level of quantification of 0.05% fetal RBC when screening $2 = 10^{6}$ maternal erythrocytes, as described by Porra et al. (2007) [18].

Data were acquired and analyzed using the Statistical Package for the Social Sciences (SPSS, Munich, Germany) software version 15.0. Pre-ECV data as well as post-ECV data were acquired in a dichotomous fashion and statistically analyzed using the x^{2}-test.

Before ECV

	2	1	0
Amniotic fluid index (cm)	>7 / 44	1-6 / 4	0 / 0
Placenta	Posterior / 12	Anterior / 22	Side / 13
Position	Dorsolateral / 34	Dorsoanterior / 10	Dorsoposterior / 3
Umbilical cord enlacement	None / 38	Once / 9	Twice / 0
Estimated fetal weight	<3000 g / 31	3-4000 g / 15	>4000 g / 1
Parity	Multiparous / 2	Parous / 14	Nulliparous / 31
Breech mobility	Movable / 11	Intermediate / 28	Fixed / 8
Uterine tone	Soft / 33	Contractions (rare) / 14	Contractions (frequent) / 1

Score / Number of patients

After ECV

	Yes	No
Pressure on placenta	24	25
Tokolysis	10	40
Unsuccessful ECV	19	31
CTG decelerations	4	46
Pain	12	38

Figure 1 Clinical and sonographic parameters surveyed before external cephalic version (ECV) and procedure related risk-factors documented after ECV.

Results

FMH volumes were evaluated for all 50 women with breech presentation at term that opted for a trial of ECV, both before and after the procedure. For technical reasons, clinical pre-ECV data were not available for three women, all of whom had - 0.05% fetal RBC (HbF^{+}/CA^{-}) before and after ECV. A percentage of fetal RBC of 0.05% defined the lower limit of quantification of our flow cytometric test method. This was determined in 124 spiking experiments performed by Porra et al. and confirmed in our laboratory in five independent experiments [18]. When screening $2 = 10^{6}$ erythrocytes via flow cytometry with this method out of 50 blood samples, 26 had quantifiable amounts of fetal RBC before ECV, with 0.49% being the maximal percentage.

Whereas all of our blood samples contained equal or greater amounts of fetal RBC after ECV, as compared to before the procedure, only three procedure-related increases were elevated above 0.05% (0.06%; 0.08%; 0.1%) (Figure 2). No procedure-related parameter predicted this event, and no fetal compromise was noted in spite of an estimated FMH of 80 mL. In our study population, none of the five predefined procedure-related risk factors or the eight parameters surveyed before ECV (Figure 1) turned out to be an independent risk factor for the small amount of FMH in the three cases with) 0.05% fetal RBC, as calculated by a x^2-test.

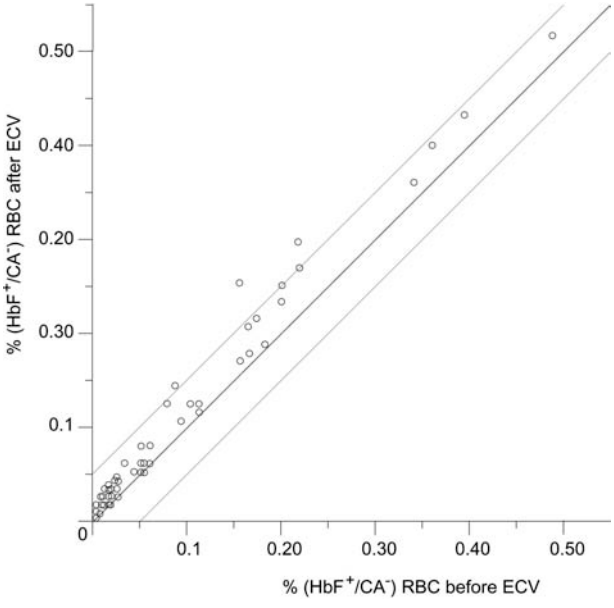

Figure 2 Dot plot of the percentage of fetal hemoglobin (HbF) positive/carbonic anhydrase (CA) negative red blood cells (RBC). Every circle represents one patient and the results of two blood samples drawn before and after external cephalic version (ECV). Gray lines indicate " 0.05% change, black line " 0 change after ECV.

Discussion

Several methods to quantify FMH are currently available [15]. KBT is a time-honored method that is still used in many laboratories, but has been shown to have a considerable intra- and inter-test variability and to lack accuracy to quantify FMH, especially at concentrations of - 1% fetal cell in maternal peripheral blood [4, 8, 13]. Furthermore, KBT is prone to overestimate FMH due to the persistence of HbF, which is especially prominent in patients with hemoglobinopathies, but is also present in up to 25% of all healthy women in the second trimester of pregnancy [14, 17]. Monoclonal fluorochrome-labeled antibodies have been implemented, either in fluorescence microscopy or in flow cytometry, which has been shown to be more sensitive compared to KBT [9]. Fluorescence microscopy has been shown to be equally precise compared to flow cytometry – which remains the gold standard for quantification of low concentrations of FMH, but utilizes less expensive equipment [12, 15]. Other techniques like the gel agglutination test (GAT) have a role in determining an amount of fetal erythrocytes in maternal circulation that exceeds the standard dose of anti-D immune globulin administered in clinical practice but are less suitable to detect low concentrations of FMH [6]. In the same context, dual-color flow cytometry using anti-HbF and anti-D monoclonal antibodies has been recently evaluated to simultaneously D-type and assess optimal doses of anti-D immunoglobulin in a single assay [19]. In our experiment, we chose a test kit that uses anti-HbF and anti-carbonic anhydrase Typ II (CA) antibodies within an established and tested dual-color flow cytometric approach to estimate as precisely and sensitively as possible the low concentrations of fetal erythrocytes expected in our study population [18]. Staining HbF, as in our test method, instead of the D-antigen for discriminating fetal erythrocytes, offers the advantage of being applicable regardless of the blood group of the mother or the fetus. A subpopulation of adult erythrocytes, however, contains HbF. These so-called F-cells are usually discriminated by their slightly lower expression of HbF compared to fetal erythrocytes. This somewhat arbitrary cut-off level can be difficult to define, especially in cases with large amounts of F cells [7]. To add specificity and to circumvent the need for an arbitrary cut-off point, a second monoclonal antibody was added to the test kit used in our study, staining for CA, which is part of the respiratory chain and is fully expressed only after birth [1, 3]. In their study of 124 spiking experiments, Porra et al. were able to confirm a lower level of quantification of 0.05%, which we were able to reproduce in concomitant controls. With this test kit the percentage of women with) 0.05% fetal RBC even before ECV was higher than previously reported [18]. Given that we confirmed our level of quantification at 0.05% in preliminary experiments as well as in concomitant controls, it is highly

unlikely that any subtle alterations made by the manufacturer to improve the test kit might account for this difference. Since all of our controls confirmed that the test method yielded accurate results, we must assume that our study population happened to have physiologically elevated levels of fetal RBC. There is some scientific debate on how to estimate transfused fetal blood volume from the percentage of fetal RBC in maternal circulation, and where to draw the line between physiological values and clinically relevant FMH [16]. For flow cytometric analysis, 0.85% fetal RBC is considered to be equivalent to 30 mL fetal whole blood, which is the volume commonly used to define clinically relevant FMH [16]. All of the blood samples tested in this study were substantially below this threshold before and after ECV. These data, generated by screening $2 = 10^6$ erythrocytes via flow cytometry, were in line with the findings of Boucher et al., who found a single FMH after ECV among 1311 women screened by single-slide manual KBT with 400 = magnification [2]. Boucher et al. reported a total of 40 out of 1311 women as having an increase in RBC volume after ECV. Given the large study population, their data are likely to represent the true incidence of FMH after ECV. Our cytometry data provide further assurance about the safety of ECV, given that quantifiable percentages of fetal RBCs were only found in three of 50 women, even in a population with rather high physiologic levels of fetal RBC before ECV and with a highly precise test method. Blood samples of these three women barely crossed the lower limits of quantification.

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Effects of Progesterone and Its Antagonist Mifepristone on Progesterone Receptor A Expression in Human Umbilical Vein Endothelial Cells

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Key Words
Human umbilical vein endothelial cells · Progesterone receptor A and B · Progesterone receptor antagonist

Abstract
Effects of female steroid hormones on endothelial cells are gaining increased importance due to several studies on the effects of hormonal treatment on cardiovascular risk. Recent data argue for an improvement of endothelium-derived relaxation and impaired vascular contraction by estradiol, whereas progesterone and testosterone might entail contrary effects. So far, gestagenic influence on endothelial cell physiology is poorly understood. Human umbilical vein endothelial cells (HUVECs) exposed to the female sex hormones estradiol and progesterone show expression of estrogen receptor-β (ERβ) and progesterone receptor A (PR-A), and are negative for ERα and PR-B. The aim of this study was to analyze the expression and stimulation of PR-A and -B in HUVECs after stimulation with progesterone and PR antagonists that are commercially available. PR-B expression or upregulation was abrogated after application of progesterone or antagonists to HUVECs. Expression of PR-A could be significantly upregulated with progesterone and mifepristone. Unexpectedly, stimulation with the progesterone antagonist RU486 (mifepristone) was accomplished by an upregulation of PR-A expression in our study. We conclude that gestagenic effects on HUVECs independent of modulators are mediated via the PR-A.

Introduction

Vascular endothelial cells are involved in the regulation of angiogenesis, inflammatory responses, vascular tone and permeability. Impaired endothelial function leads to increased cardiovascular risk [1]. In females, endothelial dysfunction gradually ensues after the menopause [2], and is associated with disturbed dilatation [3], decline in endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) bioavailability as well as abnormal endothelial morphology [4, 5]. These functional alterations of endothelial cells contribute to the increased risk of cardiovascular diseases seen in postmenopausal women [6]. The incidence of coronary heart disease (CHD) in premenopausal women is significantly lower

B.T. and C.S. contributed equally to this study.

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than in age-matched men with similar risk profiles and increases after menopause [7–9].

With regard to hormonal treatment (HT) in postmenopausal women, progesterone is generally co-administered with estrogen to prevent endometrial cancer by opposing the proliferative effect of estrogens. So far, the influence of the administered progestagens on cardiovascular function and development of atherosclerosis remains controversial [10, 11]. However, HT has been regarded as an effective tool to protect postmenopausal women from CHD [12]. Until now, major randomized clinical trials have failed to confirm the cardiovascular advantages of HT [13].

The Heart and Estrogen/Progestin Replacement Study showed that the co-administration of medroxyprogesterone acetate (MPA) with conjugated equine estrogen (CEE) did not reduce the rate of events in postmenopausal women with established CHD, yet the treatment did increase the rate of thromboembolic events and gallbladder disease [14–18].

The Women's Health Initiative trial showed that HT combined with CEE and MPA was associated with a nonsignificant increase in CHD in postmenopausal women, whereas women in the sister cohort, receiving CEE alone, showed a nonsignificant decrease in coronary events, along with a significant reduction in a composite outcome of CHD events in younger women [19, 20].

However, progesterone or other synthetic progestins have variable influences on endothelial function. For example, natural progesterone increases endothelial NO production, whereas MPA is devoid of such action [21]. In nonhuman primates, MPA has been shown to interfere with the atheroprotective effects of estrogens, which was not encountered with natural progesterone [22, 23]. In support of these observations, discrepant effects of progestins have also been described in other tissues [24].

A diversity of progesterone receptor (PR) activators and inhibitors exists with different potential to bind to PR-A, PR-B or both. In our study, we analyzed the expression of PR-A and PR-B in human umbilical vein endothelial cells (HUVECs) after stimulation with progesterone and its antagonist mifepristone with specific monoclonal antibodies by immunocytochemistry.

Material and Methods

Cell Culture

HUVECs were obtained from Promocell (Heidelberg, Germany) at passage 2 or 3. The cells were cultivated in phenol red free endothelial cell growth medium (ECGM) (Customer formulation, Promocell, Heidelberg, Germany). ECGM contained 10% fetal bovine serum, 1.0 mg/ml hydrocortisone, 0.1 ng/ml endothelial growth factor, 1.0 ng/ml basic fibroblast growth factor and 2 ml endothelial cell growth substrate as well as 5 ml streptomycin (Biochrom AG, Berlin, Germany) and 5 ml amphotericin B (Biochrom) in 500 ml medium.

HUVECs were used for experiments between passages 3 and 4. All cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C. HUVECs were incubated with progesterone and mifepristone in different concentrations (0–100 nmol/ml; table 1) and cultivated for up to 72 h on chamber slides (Nunc, Wiesbaden, Germany). These concentrations are related to physiological progesterone concentrations in pregnant human females. Cells were fixed with methanol/ethanol (50/50%; Merck, Darmstadt, Germany). The Human Investigation Review Board of the Ludwig Maximilian University Munich approved the study.

Table 1. Substances used for stimulation of PR-A

Substance	Dilution	Source
Progesterone, nmol/ml	0.1–100	Sigma-Aldrich
Mifepristone, nmol/ml	0.1–100	Roussel–Uclaf

Immunocytochemistry

Expression of PR-A was analyzed by using a specific monoclonal antibody (1A6, Mouse IgG1, 1:50, Immunotech, Prague, Czech Republic) and the ABC staining method (Vectastain Elite mouse-IgG-Kit, Vector, Burlingame, Calif., USA). Staining intensity was graded by using a semiquantitative score by counting the absolute percentage of positive-stained cells.

Two blinded, independent observers evaluated the specific immunocytochemical staining reaction without knowing the prior evaluation of each specimen. In each condition, 6–9 independent specimens were taken and evaluated independently.

In brief, HUVECs were cultivated under sterile conditions in chamber slide cultures Quadriperm (Nunc) for up to 72 h, dried, wrapped and stored at –80°C as described earlier [25]. After thawing, cells were briefly fixed with formalin (Merck; 5% in PBS, 5 min). Slides were incubated in methanol/H_2O_2 (30 min) to inhibit endogenous peroxidase activity, washed in PBS (5 min) and treated with goat serum (20 min, room temperature, RT) to reduce nonspecific background staining. Incubation with the primary anti-PR-A antibody (1A6, Mouse IgG1, 1:50, Immunotech) was done overnight at 4°C. Sections were then incubated with the biotinylated secondary anti-mouse antibody (1 h, RT) and avidin-biotinylated peroxidase (45 min, RT). Between each step, the sections were washed with PBS (pH 7.4), three times. Peroxidase staining reaction was done with diaminobenzidine/H_2O_2 (1 mg/ml; 5 min) and stopped in tap water (10 min). Sections were counterstained in hematoxylin (1 min) and then coverslipped. In controls, the primary antibody was replaced with preimmune mouse serum. Positive (MCF-7 breast cancer cell line) and negative control cells (MDA-MB231), both from American Type Culture Collection (Manassas, Va., USA), for PR-A staining were always included. The slides were finally

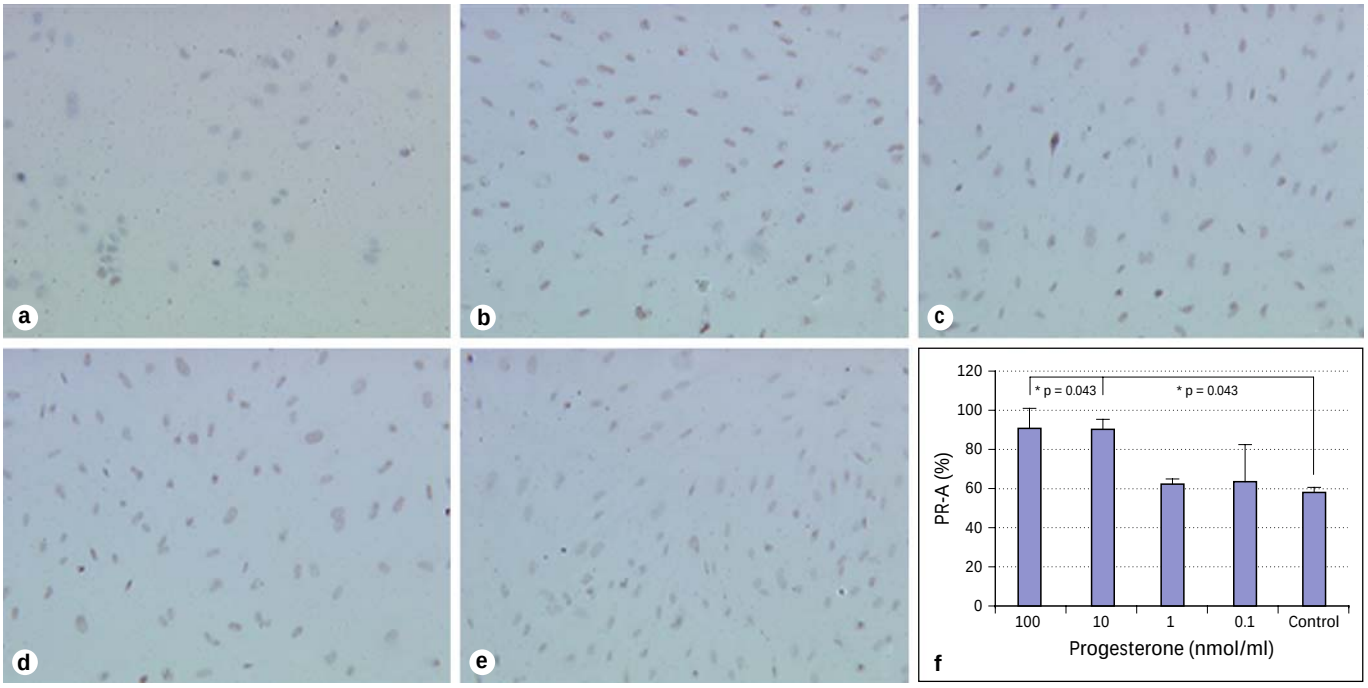

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Fig. 1. a PR-A expression in unstimulated HUVECs. ! 10. **b** PR-A expression in HUVECs after 0.1 nmol/ml stimulation with progesterone. ! 10. **c** PR-A expression in HUVECs after 1.0 nmol/ml stimulation with progesterone. ! 10. **d** PR-A expression in HUVECs after 10 nmol/ml stimulation with progesterone. ! 10. **e** PR-A expression in HUVECs after 100 nmol/ml stimulation with progesterone. ! 10. **f** PR-A expression in HUVECs after incubation with progesterone.

embedded in mounting buffer and examined with a Zeiss Axiophot photomicroscope (Carl Zeiss, Jena, Germany). The extent of PR expression was determined in a blinded fashion in one run with identical staff, equipment, and chemicals.

From each section, 5 digital pictures were taken at random of different places of stained HUVECs (! 200 magnification; 3CCD color camera; Axiocam) and examined with a Zeiss Axiophot photomicroscope.

Statistics

The SPSS/PC software package (SPSS, Chicago, III., USA) version 15.0 and 16.0 was used for collection, processing, and statistical data analysis. Statistical analysis was performed using the nonparametric Mann-Whitney U signed rank test for comparison of the means. p ! 0.05 values were considered statistically significant.

Results

Progesterone

Progesterone acts as a natural PR ligand (PR-A, PR-B). HUVECs stimulated with 100 pmol/ml and 1, 10, and 100 nmol/ml progesterone, respectively, showed expression of PR-A after cultivation for up to 72 h (fig. 1a–e). Significantly elevated PR-A expression could be observed by administration of 10 and 100 nmol/ml progesterone, as described (p ! 0.05; fig. 1f).

Mifepristone

Unexpectedly, administration of the PR antagonist RU486 led to an upregulation of PR-A expression. HUVECs stimulated with 1, 10, and 100 nmol/ml mifepristone showed significant upregulation of PR-A in all cases compared with nonstimulated controls (p ! 0.05, respectively; fig. 2).

Discussion

Recently, basic findings on the expression of estrogen receptor (ER) and PR in HUVECs were published, indicating the lack of ERα and PR-B expression in HUVECs [25].

In our study, we were able to demonstrate that progesterone acts as an activator on endothelial cells and is able

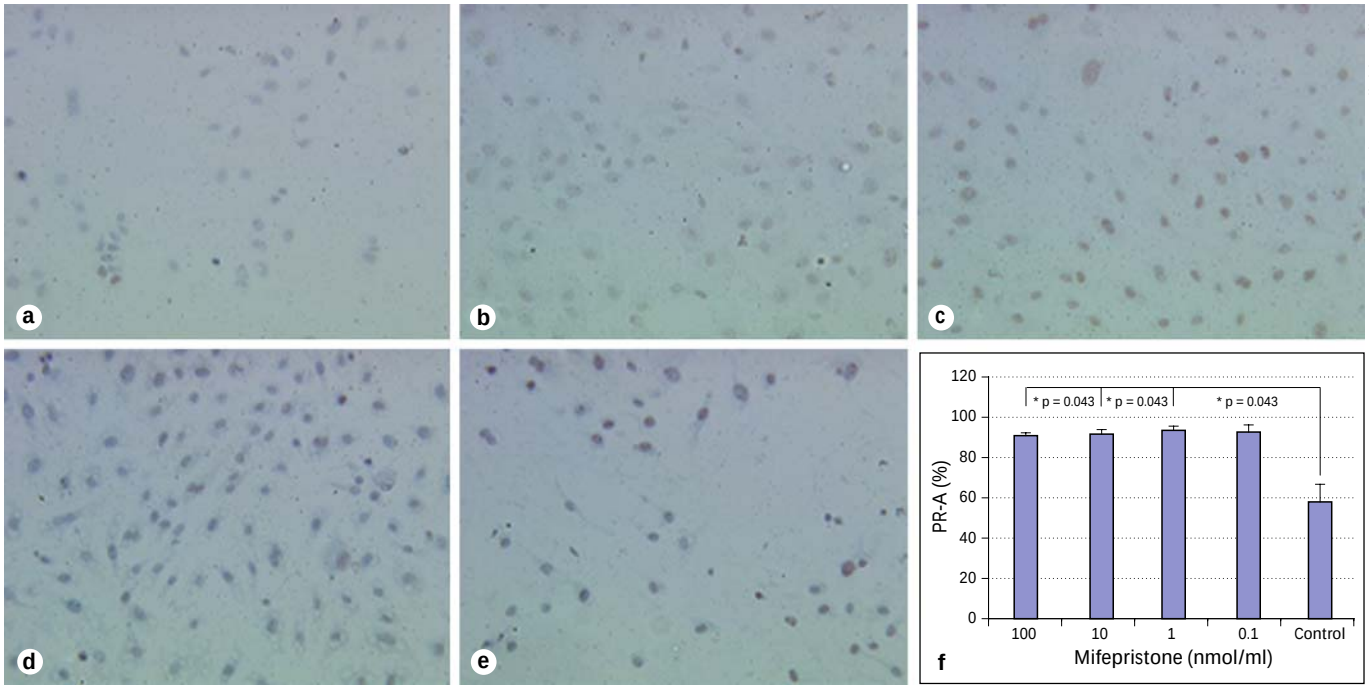

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Fig. 2. a PR-A expression in unstimulated HUVECs. ! 10. **b** PR-A expression in HUVECs after 0.1 nmol/ml stimulation with mifepristone. ! 10. **c** PR-A expression in HUVECs after 1.0 nmol/ml stimulation with mifepristone. ! 10. **d** PR-A expression in HUVECs after 10 nmol/ml stimulation with mifepristone. ! 10. **e** PR-A expression in HUVECs after 100 nmol/ml stimulation with mifepristone. ! 10. **f** PR-A expression in HUVECs after incubation with mifepristone.

to upregulate PR-A expression in a dose-dependent manner. In contrast to former studies, PR antagonist RU486 also led to an upregulation of PR-A expression.

The cellular effects of gestagens are mediated by binding to nuclear receptors (PR) which activate transcription of genes involved in cellular growth control. So far, endothelial effects of gestagens are poorly understood and, as compared with data on estrogenic influences on endothelial cells, also poorly investigated. Fu et al. [26] investigated effects of progesterone and MPA on actin remodeling, moesin activation and cell movement in human endothelial cells. They were able to show that both gestagens regulate endothelial cell movement by rapidly signaling to the actin-binding protein moesin and to the actin cytoskeleton.

To further study the effects of gestagens on vascular function, Hermenegildo et al. [27] studied the effects of progesterone and MPA on prostacyclin production in HUVECs. Both gestagens significantly increased prostacyclin release in a time- and dose-dependent manner by enhancing Cox-1 and Cox-2 expression and activities.

In contrast to our recent findings on the lack of PR-B expression on HUVECs, Tatsumi et al. [28] described PR-A and PR-B mRNA expression on HUVECs. The authors investigated the effect of progesterone, MPA, norethindrone acetate, levonorgestrel as well as dienogest on cytokine-stimulated HUVEC expression of adhesion molecules. However, progesterone or dienogest did not affect IL-1β-stimulated ICAM-1 or VCAM-1 expression, whereas the other gestagens did.

Additionally, concomitant addition of mifepristone blocked the gestagen-induced increase in adhesion molecules. They concluded that dienogest unlike other synthetic progestins lacks the stimulatory effect on cell adhesion molecules [28]. We were able to show that HUVEC stimulation with increasing amounts of RU486 leads to an upregulation of PR-A in a dose-dependent manner. Our data implicate that RU486 only acts as a PR antagonist in the presence of PR activators like progestone.

The Women's Health Initiative trial reported an excess of heart diseases in postmenopausal women receiving MPA. Therefore, Simoncini et al. [21] investigated the effects of progesterone, MPA, dydrogesterone and its me-

tabolite 20-a-dihydrogesterone on endothelial synthesis of NO, and characterized the signaling events recruited by these compounds. In contrast to dydrogesterone, progesterone and 20-a-dihydrogesterone, MPA did not trigger eNOS enzymatic activation and decreased the extent of eNOS induction by estradiol. The authors concluded that their findings support the concept that synthetic progestins act differently on vascular cells and that hormonal preparations may differ in their cardiovascular effects [29].

Studies on the effects of HT in postmenopausal women indicate procoagulant effects of gestagens and estrogens. To further study the effects of gestagens on hemostasis, Zerr-Fouineau et al. [30] investigated whether progestins affect the formation of NO in endothelial cells and examined the underlying mechanism.

Certain progestins, including MPA, reduced the antiaggregatory effect of endothelial cells by decreasing the expression of eNOS and the formation of NO in endothelial cells; an effect that is mediated via activation of glucocorticoid receptors.

In summary, our study showed that progesterone is able to upregulate PR-A expression in a dose-dependent manner and that the PR antagonist mifepristone also acts as a PR activator when administered solely.

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Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical expression of glycodelin in breast cancer correlates with estrogen-receptor α and progesterone-receptor A positivity

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Summary. Glycodelin (Gd), previously known as placental protein 14 (PP 14), acts as an immunosuppressive glycoprotein by suppressing the cytolytic capacity of human natural killer (NK) cells and T-cells *in vitro*. Glycodelin is expressed in normal glandular epithelium of the endometrium as well as in normal and malignant glandular cells in and outside of the reproductive tract. Recently, Gd expression was demonstrated in normal and cancerous human breast tissue.

Paraffin-embedded breast cancer tissue blocks (n=121) were examined for Gd expression. No part of the specimens contained carcinoma *in situ*.

Gd expression was present in lobular and ductal breast carcinoma. We observed expression of Gd in breast cancer independent of grading. With regard to nodal status, no significant differences in the expression of Gd between cancer tissue from patients with or without axillary lymph node metastases were present. However, Gd expression was found to be significantly higher in breast cancer tissue when the staining reaction for steroid receptors was also positive.

These results implicate that Gd might be an additional marker for the differentiation of breast cancer tissue. To which extent Gd could serve as an additional indicator for breast cancer survival is part of our ongoing research.

Key words: Glycodelin, Breast cancer, Differentiation, Immunhistochemistry

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Introduction

Breast cancer is the most common malignant tumour in women worldwide. Due to constantly enhanced therapeutic regimens its survival rates have improved substantially over the last 20 years. Hormone sensitivity is the most important predictive marker, indicating better prognosis due to available endocrine treatment options. In addition, hormone receptor positivity in breast cancer cells indicates better differentiation and less aggressive malignant behaviour. Treatment of early stage breast cancer depends on endocrine responsiveness, which is defined as staining steroid-receptor positive in more than 10% of breast cancer cells (Goldhirsch et al., 2007).

However, the biological significance of low-level expression of steroid receptors in breast cancer cells remains an area of scientific concern (Payne et al., 2008). Additional markers that are associated with better differentiation and intracellular signalling are needed in order to tailor endocrine therapy to those cancer patients that are likely to respond. The immunoreactivity of glycodelin (Gd) was evaluated in invasive breast cancer specimens (n=121) to investigate the capability of Gd as a marker of differentiation.

Glycodelin, a member of the family of lipocalins, is a secreted glycoprotein found in variable concentrations and tissue specific glycosylation in the human genital tract and is known to be expressed in breast cancer (Kamarainen et al., 1999). It exists in various isoforms with different glycosylation patterns that influence their staining behaviour (Morris et al., 1996; Tse et al., 2002; Chiu et al., 2007). The structure of a specific breast cancer glycodorm remains to be investigated. Nevertheless, invasive breast carcinoma without axillary or distant metastases were found to have a higher expression of Gd, hinting towards a better differentiation

and prognosis (Jeschke et al., 2005). Kämäräinen et al. (Kamarainen et al., 1997) were able to show that glycodelin expressing cells also upregulated markers of organized epithelia, such as cytokeratins 8 and 18, as well as E-cadherin, and physiologic changes in intracellular distribution of ß-catenin. This expression-pattern was accompanied by the acquisition of a phenotype of organized glandular epithelium.

Gd is further known to have direct immuno-modulatory effects by suppressing the reactivity of stimulated T- lymphocytes and by inhibiting NK- and B-cell function *in vitro* (Seppala et al., 2002). Therefore, Gd might contribute towards tumour progression in an early phase of carcinogenesis in which organized local immune evasion is provided by the expression of Gd, while intracellular signalling is still intact.

Material and methods

Specimens

Formalin-fixed paraffin-embedded tissue from breast cancer patients undergoing surgery at the First Department of Obstetrics and Gynaecology of the Ludwig- Maximilians-University of Munich were investigated. All specimens had a histological classification as lobular or ductal breast cancer by a gynaecological pathologist. Patients with metastatic disease were excluded, as well as all patients with any proportion of ductal carcinoma *in situ* (DCIS) within tumorous tissue, as Gd expression has been seen in virtually all cases of DCIS (Jeschke et al., 2005).

Tissue blocks of 121 breast cancers fulfilled the above mentioned criteria. The mean age of the patients was 62,2 (SD 9,8) years (45-84 years) with 85% being postmenopausal. Out of the 83 ductal carcinoma 9 were graded as “G1”, 40 as “G2” and 34 specimens as “G3”. With regard to lobular carcinoma (n=38) 15 were graded as “G1”, 16 as “G2”, 3 as “G3”. Grading was performed according to criteria published by Elston and Ellis (Elston and Ellis, 2002). If one or more of the criteria was not assessable due to technical variations the specimen was classified as Gx, as was the case in 4 lobular carcinoma specimens. Nodal involvement, as well as hormone receptor status was equally distributed among the study population. As part of the clinical work-up ER-α and PR-A isoforms are evaluated. In our study population all “receptor-positive” breast cancer specimens were positive for ER-α and PR-A. (ER-α and PR-A positive: 48 ductal, 20 lobular; ER-α and PR-A negative: 35 ductal, 18 lobular).

Immunohistochemistry

Immunohistochemical detection of Gd expression on paraffin sections (4 µm) of breast cancer tissue was performed according to procedures described previously (Jeschke et al., 2005). In a first step sections were incubated in 3% methanol/ H_2O_2 (30 min) to inhibit endogenous peroxidase activity, washed in phosphate-buffered saline (PBS, pH 7.4) for 5 min and treated with 1.5% goat serum for 20 min at 22°C to reduce non-specific background staining. To loosen aldehyde bonds sections were boiled in an acidic buffer (pH6.0) containing 2% Na-citrate and citric acid for 5 min and subsequently cautiously cooled to room temperature in tap water. The primary monoclonal antibody, known to recognize the CH epitope on Gd was purchased at Glycotope GmbH (Berlin, Germany) (code: A87-B/D2; isotype: $IgG1_\kappa$) has been characterized previously (Jeschke et al., 2006). Specific binding of the antibody to Gd was analyzed by Western blot analysis. To specify immunoreactivity against the A isoform of glycodlin we included a seminal vesicle (known to express glycodlein S, which is the other most common Gd isoform) as a negative control (Fig. 1C). Incubation with the primary antibody was performed overnight at 4°C. Sections were then incubated with biotinylated secondary anti-mouse antibody (1:200) for 1 hr and then with avidin-biotin peroxidase complex (1:25) (ABC) prepared according to manufacturer’s instructions for 45 min (Vectastain-Elite-ABC Kit, Vector Laboratories, Burlingame CA, USA). Labelling was revealed by incubation with 1 mg/ml diaminobenzidine/H_2O_2 (Dako, Hamburg, Germany) for 5 min and the reaction stopped by washing in tapwater for 10 min. Sections were counter-stained in hemalaun for 1 min, then cover-slipped. The primary antibody was replaced with pre-immune rabbit serum in controls. Positive (decidual tissue) and negative (human trophoblast tissue) controls were always included.

Two independent observers, including a gynaecological pathologist, assessed the specimens using the semi-quantitative immunoreactive score (IRS) after Remmele and Stegner - routinely used for assessing receptor positivity in breast cancer - evaluated the intensity and distribution patterns of the staining reaction. The IRS was calculated by multiplication of optical staining intensity (graded as 0=no, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive stained cells (0=no staining, 1≤ 10% of the cells, 2=11-50% of the cells, 3=51-80% of the cells and 4≥ 81% of the cells). Evaluation of each specimen was performed without having any knowledge of the pathological diagnosis. For all sections, we assessed the mean optical density and the quantity of pixels which had a positive reaction for glycodelin using the KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Germany)

Statistics

The SPSS/PC software package, version 16.0 (SPSS GmbH, Munich, Germany), was used for collection, processing and statistical analysis of all data. Statistical analysis was performed using the non-parametrical Mann-Whitney U test and in case of 3 or more groups its extension, the Kruskal-Wallis one-way analysis of variance by ranks. All p-values resulted from two-sided statistical tests and $p \leq 0.05$ was considered to be significant.

Results

Characteristics of study population

121 breast cancer specimens were included in the study. 83 of them were classified as ductal carcinoma, 38 as lobular. Within those subgroups grading was distributed as follows: 89% (n=74) of ductal carcinoma were classified being G2 or G3, whereas for lobular carcinoma 81% (n=38) were graded as G1 or G2 (Table 1).

Glycodelin expression

Gd was expressed both in lobular and in ductal breast carcinoma. Although there was a tendency towards increased staining in ductal carcinoma, no significant differences were found (median IRS for ductal carcinoma: 1.81 versus median IRS = 1.55 lobular carcinoma) (Fig. 1A). Gd expression in lobular and ductal carcinoma cells was independent of their grading (Fig. 1B). Fig. 2C shows staining examples of a Gd negative (IRS:0) lobular G1 breast cancer specimen and a Gd positive (IRS:4) ductal G2 specimen. Although not statistically significant, there has been a tendency

Table 1.

	ductal	lobular
G1	9	15
G2	40	16
G3	34	3
Gx	0	4
Σ	83	38

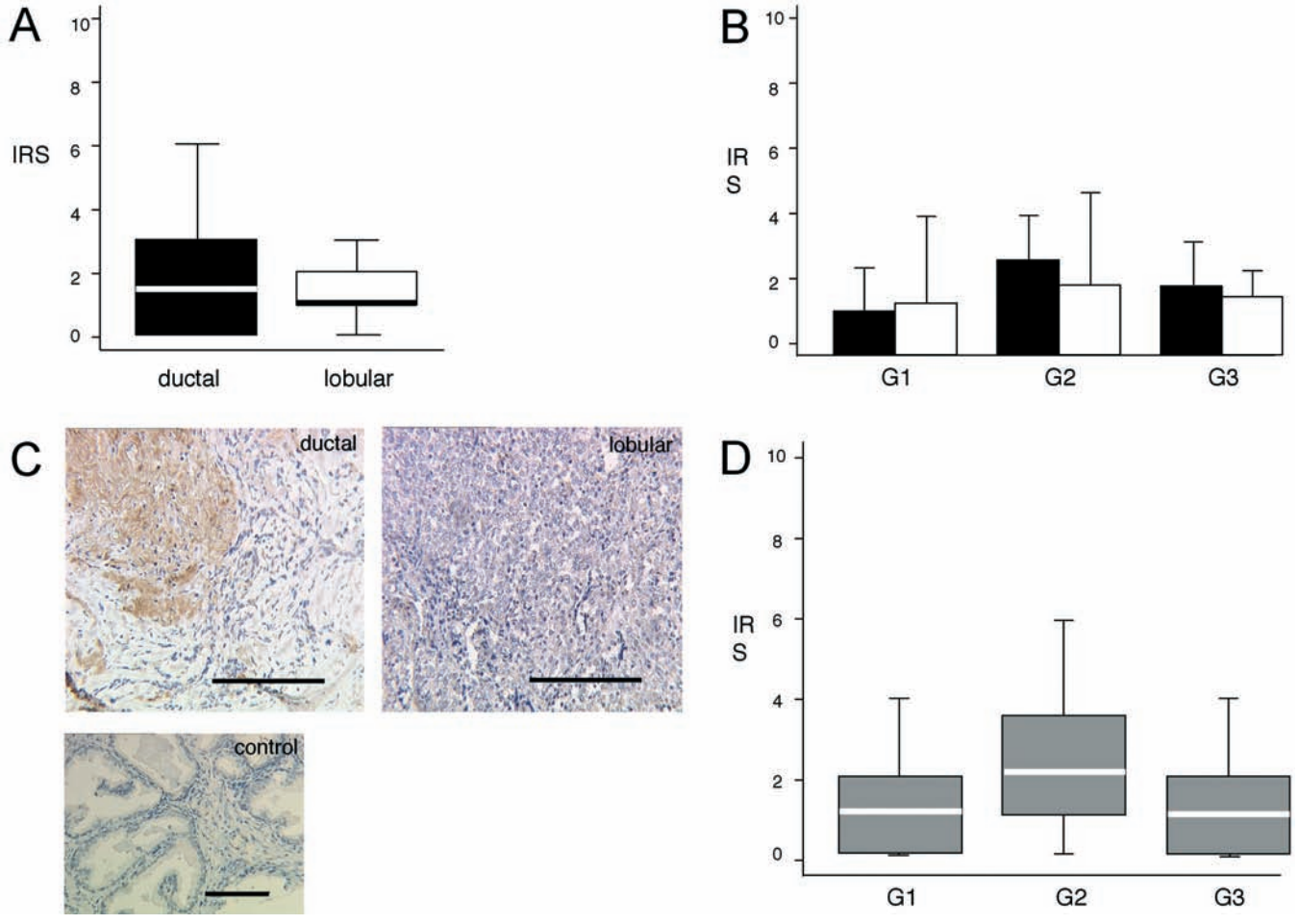

Fig. 1. Glycodelin is equally expressed in ductal and lobular breast cancer and its expression is independent from grading. Immunohistochemical evaluation according to the Immune Reactivity Score (IRS) revealed no significant difference between ductal and lobular breast cancer specimens **(A).** Two staining examples and a negative control (seminal vesicle) are shown in **(C)**. For box plots boxes indicate 25th and 75th Quartile with horizontal bars indicating median staining intensity. Error bars denote standard error of mean (SEM) (**A, B, D**). No statistically significant difference between different grading characteristics could be determined for ductal and lobular breast cancer (**B**). Summative results revealed a tendency towards higher expression in G2 tumours yet no statistically significant difference (**C**). Bars: 200 µm.

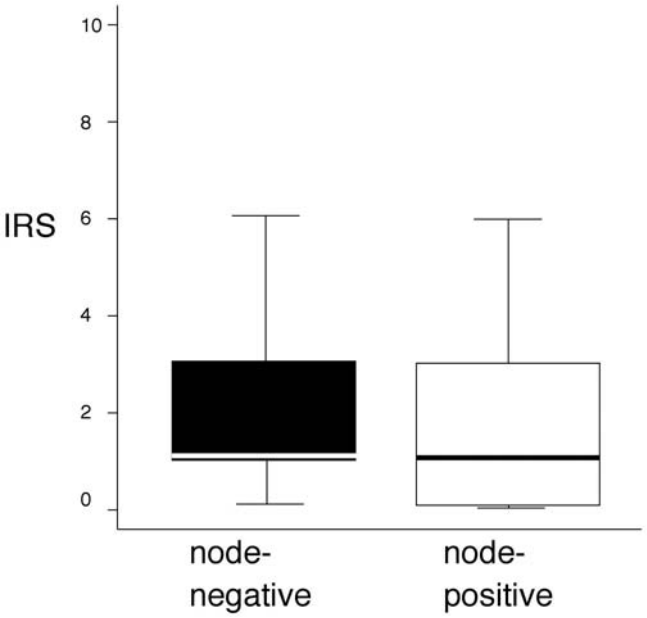

Fig. 2. Glycodelin expression of breast cancer dependent on nodal status. Patients with positive axillary lymph nodes showed no significantly higher Gd expression.

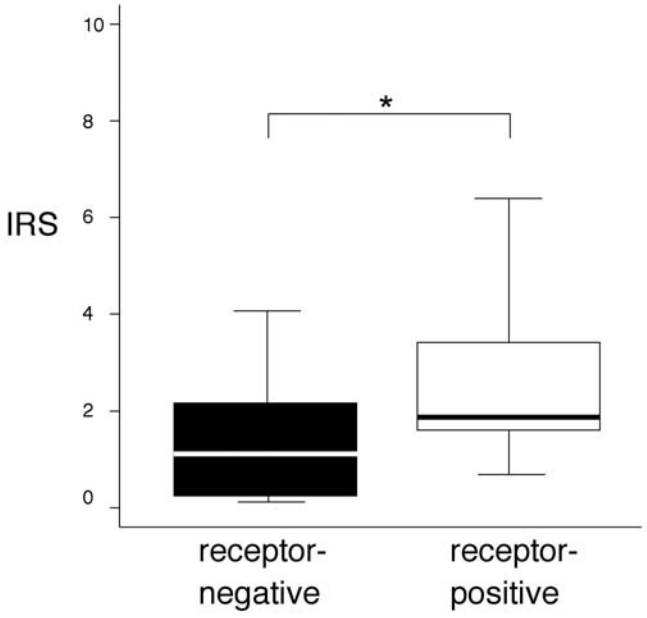

Fig. 3. Glycodelin expression in breast cancer was significantly higher in tissue specimens that are receptor positive as compared to receptor negative specimens ($p<0,05$). Receptor negative specimens (n=53) were compared to receptor positive specimens (n=68) and statististical significance of histochemical reactivity against Gd was determined by the Mann-Whitney-U test.

towards higher expression of Gd in G2 tumours in a pooled analysis of lobular and ductal carcinoma (Fig. 1D)

There was no significant difference between tumours of patients with positive axillary lymph nodes and those without. Median IRS for tumours without nodal involvement was 1.59 (n=61) for those with affected lymph nodes 1.87 (n=60) (Fig. 2).

Gd expression was found to be significantly higher in those specimens that also stained positive for steroid receptors. Median IRS of all hormone receptor negative tissue sections was 1.4 (n=53) compared to hormone receptor positive specimens that had a median IRS of 1.99 (n=68) ($p=0.033$).

Discussion

Gd expression was analysed in breast cancer tissue without any parts of the specimens containing carcinoma *in situ*. The monoclonal antibody used in these experiments detects specific carbohydrate structures of Gd (Jeschke et al., 2006). Glycosylation seems to play a pivotal functional role in Gd (Seppala et al., 2007). We found a significant increase in glycosylated Gd protein expression in hormone receptor positive (ER-α and PR-A) specimens. However, Gd expression was independent of nodal involvement, grading and cancer type. To our knowledge this study is the largest done so far on Gd expression in breast cancer. Kämärrainen et al. (1999) were able to describe Gd expression in 21/35 ductal carcinomas, 9/9 tubular carcinomas, 9/9 mucinous carcinomas, 3/3 mixed ductal/tubular carcinomas and 7/11 lobular carcinomas However, no differentiation was made according to ductal carcinoma in situ (DCIS). Later on, it became clear that DCIS uniformly stained positive for Gd (Jeschke et al., 2005). Therefore, we excluded all specimens that contained any fraction of DCIS in addition to invasive cancer. In accordance to the study population used by Kämärrainen et al. we did not find any correlation of Gd expression and nodal involvement (Kamarainen et al., 1999). The pivotal clinical marker of differentiation is “Grading”. Grading of our specimens was performed by a gynaecological pathologist according to the most commonly used international criteria by Elston and Ellis (Elston and Ellis, 2002). To our knowledge the influence of grading on Gd expression has never been tested before.

Surprisingly, no significant differences between grading types was seen. A tendency towards higher levels of Gd expression in G2 tumours only in a pooled analysis might be explained by the immunomodulatory properties of Gd. Antigenic properties of tumours are altered during carcinogenesis. Secretion of a glycoprotein that inhibits NK- and T-cell responses locally would be more important for earlier tumour stage survival. We speculate that in later stages of differentiation this mechanism becomes less important. Nevertheless, the tendency of G2 tumours to express Gd did not reach statistical significance in our study

population.

Gd expression was found to be significantly higher in those specimens that were positive for steroid receptors. Expression of Gd is known to be dependent on steroid hormone levels. It is normally expressed in steroid responsive tissues of the reproductive tract and its secretion is strictly dependent on progesterone levels (Seppala et al., 2002). In endometrium, as well as cancerous tissue, Gd expression changes with steroid receptor notably PR expression (Mandelin et al., 2003; Stavreus-Evers et al., 2006). In our study population all 68 „receptor-positive" invasive breast cancer specimens stained postive for ER-α and PR-A in the routine clinical work-up. IN routine clinical work-up other isoforms are usually not assessed as ER-α and PR-A positivity will decide whether to start antihormonal therapy. Possible associations between Gd and other steroid receptor isoforms, as well as putative Gd isoform differences between tissue specimens could not be investigated in this study. Gd is known to be an efficient inhibitor of E-selectin mediated cell adhesion *in vitro*, fostering tumour spread (Jeschke et al., 2003). Its high expression in well-differentiated breast cancer tissue might contribute towards the clinical phenomenon of early micro-metastatic spread even in small G1 tumours. On the other hand, Gd expression is known to be associated with higher differentiation. During neoplastic progression Gd mRNA and protein levels fall, while transfection of glycodelin cDNA in breast cancer cell cultures suppressed proliferation and induced a differentiated epithelial glandular phenotype (Jeschke et al., 2003, Kamarainen et al., 1997). Taken together, our study promotes Gd as an additional marker for differentiation, though additional, larger studies, as well as carefully designed *in vitro* experiments, will be needed to show progesterone dependent differentiation and glycosylation effects on breast cancer.

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Ovarian Cancer-derived Glycodelin Impairs In Vitro Dendritic Cell Maturation

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Summary: Local immunosuppressive mechanisms shape the tumor microenvironment and contribute to carcinogenesis. In ovarian cancer such mechanisms have been shown to influence survival. Dendritic cells (DCs) are central immunity regulators and induce potent cytotoxic T-cell responses as well as peripheral tolerance depending on modulatory stimuli. Here, we show that ovarian cancer-derived glycodelin (Gd), a glycoprotein that physiologically modulates local immunity in early pregnancy, induces a tolerogenic DC phenotype. Gd was isolated with high performance liquid chromatography from the malignant ascites of ovarian cancer patients. DCs were generated from monocytes of healthy donors and exposed to Gd with or without an inflammatory stimulus (tumor necrosis factor-a and interleukin 1-b). We investigated the effect of Gd on DC surface marker expression, endopinocytotic activity, cytokine profile, and lymphoproliferative activity. DCs that were exposed to Gd altered their phenotype as seen by a differential expression of costimulatory molecules, whereas expression of DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, a marker of an immature phenotype, was increased. Functional data provided further evidence for the immature/tolerogenic properties of Gd-pretreated DCs. Antigen uptake was retained, production of interleukin-10 was increased, and lymphoproliferative activity was reduced. This effect was reversible by adding Gd-blocking antibodies. Gd, which is found in the malignant ascites of ovarian cancer patients, induces a tolerogenic phenotype in DC, thereby shaping an immunodeficient tumor micromilieu.

Key Words: dendritic cells, glycodelin, pregnancy glycoproteins, tumor micromilieu, epithelial ovarian cancer

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Human tumors employ many strategies to evade or structure host immune responses to shape a microenvironment that supports their malignant growth.[1] As such, one of the dominant mechanisms of immune evasion is tumor antigen-specific T-cell tolerance. Dendritic cells (DCs) are central immunity regulators that can either initiate primary T-cell immune responses or maintain peripheral tolerance by suppressing (auto) antigen-specific immunity.[2] In ovarian cancer, immunocompetent DC infiltration has been associated with prolonged survival, whereas immunosuppressive mechanisms are associated with disease progression and increased mortality.[3,4] Furthermore, immunogenicity toward ovarian cancer antigens has been improved by suppressing DC-mediated tolerogenic mechanisms.[5] The capacity of DCs to induce immunity versus tolerance is dependent on their state of activation, which, in turn, is influenced by exogenous factors that are present during antigen encounter.[6] ''Danger signals'' [proinflammatory cytokines such as tumor necrosis factor-a (TNF-a) and interleukin 1-b (IL 1b)] trigger DC maturation and subsequent antigen-specific immune responses.[7] DCs are employed in immunotherapy protocols against various malignancies, including ovarian cancer, because of their unrivaled capacity to initiate cytotoxic T-cell responses.[8] Other, noninflammatory signals, however, lead toward a tolerogenic DC phenotype that might be detrimental to an antitumor immune response.

Glycoproteins are part of the tumor-specific array of immunosuppressive mediators. Glycodelin (Gd), a member of the lipocalin family, is a secreted glycoprotein found in the human genital tract. Its known physiologic function is in localized immunosuppression during early pregnancy. It is secreted in high quantities by the uterine mucosa to allow the semiallogeneic blastocyst to implant into the endometrium.[9] In ovarian cancer, Gd expression is associated with dedifferentiation, angiogenesis, and reduced survival.[10,11] It is currently used as a biomarker for early detection and monitoring of recurrent ovarian cancer.[12]

Immunomodulatory Gd effects are known for many effector cells of the innate and adaptive immune system. Gd directly inhibits natural killer-cell and T-cell function, suppressing interferon-g, and fostering IL-5 and IL-4 secretion.[13–15] Monocytes express a Gd receptor and we were able to show recently that Gd derived from human pregnancy induces a tolerogenic phenotype in DC.[16,17] However, immunologic effects of cancer-derived Gd on DCs—as the central regulators of antitumor immunity—have not been tested so far.

To examine the immunomodulatory properties of ovarian cancer-derived Gd, we investigated the effect of Gd, isolated from malignant ascites, on the phenotype and functionality of monocyte-derived DCs in vitro.

MATERIALS AND METHODS

Cell Culture

Cell cultures were maintained in Roswell Park Memorial Institute 1640 (Biochrom, Berlin, Germany)

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supplemented with 2% heat-inactivated human pooled AB serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (Life Technologies, Paisley, UK), 50 U/mL penicillin, and 50 mg/mL streptomycin (both from Sigma, Munich, Germany); hereafter, this supplemented medium is referred to as complete medium. All cultures were maintained at 371C, 5% CO_2, and 95% humidity.

Isolation and Culture of Human DCs

DCs were generated from peripheral blood monocytes (PBMCs) as described.[18] PBMCs were isolated from healthy donors by Ficoll density-gradient centrifugation (Biochrom, Berlin, Germany), washed 3 times, and resuspended in complete medium. For 60 minutes, PBMCs (5×10^6/mL) were allowed to adhere in 75-cm^2 culture flasks. Nonadherent cells were removed by vigorous pipetting. After overnight incubation, cells were transferred into 6-well plates (1.5×10^6 cells/2 mL) with fresh complete medium supplemented with 1000 IU/mL granulocyte macrophage colony-stimulating factor (Leukine; Immunex, Seattle) and 500 IU/mL (IL-4 Promega, Madison). An aliquot of DCs was assessed for purity before further use. DC maturation was induced using TNF-a(1000 U/mL) (R&D Systems, Wiesbaden, Germany) and IL-1b (10 ng/mL) (Strathmann Biotech, Hannover, Germany).

Gd Purification

The Gd used in this study was purified from pooled human ovarian cancer ascites as previously described.[19,20] Ovarian cancer ascites was loaded onto a diethylaminoethyl sepharose column and fractionated on a 50 to 500 mM NH_4HCO_3 gradient followed by gel filtration and a second anion exchange chromatography step on Resource Q. Final purification was obtained by hydrophobic interaction chromatography on Octyl-Sepharose and Resource-Phe (all columns: GE Healthcare, Freiburg, Germany) (Fig. 1A). To further determine purity, isolated protein batches were silver stained as described previously.[21] Briefly, protein batches were loaded on polyacrylamide gels and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. After that the gel was fixed for 30 minutes in methanol/acetic and incubated in 10% glutaraldehyde for 30 minutes. After a washing step in deionized water, the gel was stained in silver nitrate solution for 15 minutes. After another washing step, developing solution was added and subsequently the gel was fixed when staining was satisfactory. After a final washing step the gel was photographed in a Molecular Imager (BioRad, Munich, Germany) and stored as TIFF files.

All batches were tested for purity by Western blot (goat primary antibody: sc-122291; Santa Cruz, Heidelberg, Germany) (Fig. 1C). Biotinylated antigoat secondary antibodies (PK-6105) and avidin-alkaline phosphatase complexes (AK-5000) were purchased from Vector Laboratories (Burlingame) and used according to the manufacturer's instructions. Final tagging was performed using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Sigma, Munich, Germany), yielding purple staining of labeled bands.

To exclude endotoxin contamination, batches of purified Gd were tested for TNF-a induction in PBMCs. All samples were collected from patients who underwent reduction for malignant ascites for medical reasons. The local Ethics Committee approved the obtaining and handling of all human material.

Monoclonal Antibodies and Flow Cytometry

Mouse antihuman monoclonal antibodies and the appropriate isotype controls were purchased from Becton Dickinson (Heidelberg, Germany): anti-major histocompatibility complex (MHC)-II (IgG2b), anti-CD86 (IgG1), anti-CD83 (IgG1), anti-CD14 (IgG2b),; and anti DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (SIGN) (IgG2b). As there are no tested antibodies commercially available for blocking experiments, we combined, 3 antibodies, all of which were polyclonal IgG goat antihuman (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) and applied them in an excess concentration of 200 mg/mL. Two of these antibodies are mapping for different epitopes near the *C*-terminus of Gd of human origin [(C-15): sc-12291 and (Q-13): sc-12290]. The remaining antibody is mapping for an epitope near the *N*-terminus. For fluorescence-activated cell sorting analysis, 10^5 DCs suspended in 100 mL phosphate-buffered saline were incubated with 10 mL of fluorochrome-labeled monoclonal antibodies for 20 minutes on ice. After staining, the samples were washed and analyzed immediately (FACS Calibur; Becton Dickinson, Heidelberg, Germany). Data were analyzed using CellQuest Pro software (version 5.2.1, Becton Dickinson).

Endopinocytotic Activity

Cells were washed, and endopinocytotic activity was assessed by adding fluorescein isothiocyanate (FITC)-dextran (0.5 mg/mL) (relative molecular weight 40,000) to the culture medium for 30 minutes at 371C as described previously.[22] In brief, immature, untreated DCs were incubated at 371C to serve as positive controls. On the other hand, incubation on ice abrogates endopinocytosis and was therefore used as a negative control. After 30 minutes, cells were washed 3 times to eliminate all FITC-dextran attached to the outer cell membrane, and incorporated FITC-dextran was subsequently analyzed by flow cytometry.

Lymphoproliferative Activity

DCs were harvested and cocultured in complete medium with a constant number of allogeneic nonadherent PBMCs (2×10^5/200 mL) in 96-well round-bottom microtiter plates at ratios ranging from 1:10 to 1:160. DNA synthesis was measured using the "Cell Proliferation ELISA, BrdU (chemiluminescent)" (Roche Diagnostics, Mannheim, Germany). In short, proliferating cells incorporated 5-bromo-2-deoxyuridine (BrdU) in place of thymidine over a 12-hour period. Cells were dried and fixed, and the DNA was denatured. Subsequently, anti-BrdU peroxidase-labeled antibody was added, and the immune complexes were detected by a subsequent substrate reaction. The reaction product was quantified by measuring the light emission on a luminescent ELISA reader (MRX, Dynatech Lab, Burlington) at 450 nm.

Cytokine Secretion

Cytokine secretion was analyzed by enzyme-linked immunosorbent assay. The BD OptEIA Human IL-10 and Human IL-12 (p40) enzyme-linked immunosorbent assay sets were purchased from Becton Dickinson and used according to manufacturer's instructions. Cell culture supernatants were collected 48 hours after stimulation, stored at −201C, and subsequently analyzed.

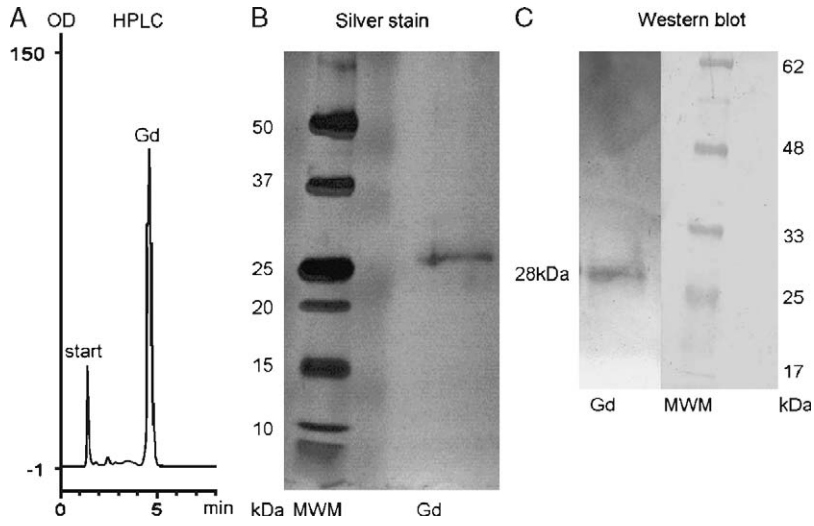

FIGURE 1. Isolation of Gd. High performance liquid chromatography (HPLC) profile with its ''start'' peak and the peak of isolated Gd. Purity of the protein was verified by silver staining, which showed a protein band at 28 kDa. Western blot analysis of purified Gd with a monoclonal antibody (A87-D /C5) verified the specificity of the isolate; MWM. Gd indicates glycodelin; MWM, molecular weight marker.

Statistical Analysis

Data are expressed as median ± SEM. Statistical significance was determined by the Student *t* test for paired samples of original values. Differences were considered statistically significant for $P < 0.05$.

RESULTS

In vitro generated immature DCs were characterized as being $CD14^-$, $CD83^-$, $CD86^-$, and having an intermediate expression of MHC class II and DC-SIGN. To test the influence of Gd on their maturation processes, DCs were incubated with 0.5 mg/mL, 10 mg/mL, or 25 mg/mL Gd over 24 hours, washed extensively, and subsequently matured using a standard combination of TNF-a (1000 U/mL) and IL-1b (10 ng/mL).[6] Surface marker expression was assessed by flow cytometry after 48 hours. Upon maturation, untreated DCs showed the expected increase in costimulatory and MHC-II molecules as well as a reduced expression of DC-SIGN. However, DCs that were subject to pretreatment with Gd had reduced expression of costimulatory and MHC-II molecules as compared with their stimulated counterparts (Fig. 2A). Incubation with Gd alone without subsequent maturation stimuli partially increased the expression of CD83 as well as DC-SIGN, leaving $CD86^-$ and MHC-II expression unchanged. In pretreated DCs, DC-SIGN mean fluorescence intensity was between matured and immature DC intensities. Interestingly, in dual parameter fluorescence-activated cell sorting-analysis, the increase in CD83 in Gd-pretreated DCs was caused by a $CD83^+$/DC-$SIGN^+$ double-positive population (Fig. 2B). To control for the specificity of this effect we added 3 polyclonal antibodies mapping for different epitopes of Gd in excess concentrations to block Gd-specific effects. By these measures the effect was partially reversible.

Similar results were obtained for DCs treated with 25 mg/mL Gd, whereas preincubating with 5 mg/mL Gd had only a marginal effect on the tested surface markers (data not shown). However, no distinct DC-SIGN-positive DC population was detected when preincubation was performed with low concentrations of Gd (data not shown). In all experiments, cell viability was assessed using the Trypan-blue exclusion method and was comparable between the different conditions and time points.

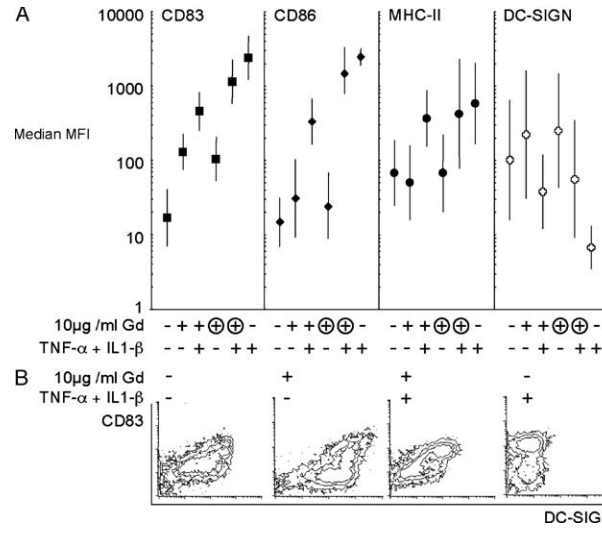

FIGURE 2. Altered expression of maturation markers and persistence of DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (SIGN) after pretreatment with 10 mg/mL Gd. DCs were pretreated with 10 mg/mL Gd for 24 hours and subsequently matured with tumor necrosis factor (TNF)-a (1000 IU/mL) and interleukin (IL)-Ib (10 ng/mL). Flow cytometry for surface markers was performed after 48 hours. Cumulative data from 9 independent experiments are shown in A for all nonblocking experiments. To control for specificity of the effect 3 blocking-experiments (indicated by a circled +) were performed. Dots represent mean fluorescence index (MFI) of all experiments, accompanied by the ± SEM. Conditions are marked by +/− as appropriate. Density plots of a representative experiment are depicted in B. DC indicates dendritic cells; Gd, glycodelin.

Immature DCs constantly take up antigen and present it via MHC molecules without concurrent costimulation. Upon maturation, however, DCs down-regulate endopinocytosis and present their antigen content together with costimulatory molecules in a stable fashion, which leads toward immunostimulation.[23] Treatment with Gd before applying maturation stimuli nonsignificantly impaired down-regulation compared with matured DCs. Incubation with Gd alone did not change endopinocytosis in a significant manner; only a marginal increase was noted compared with unstimulated conditions. Adding polyclonal antibodies to label Gd did not change this pattern (Fig. 3).

To further determine functional properties of Gd pretreated and subsequently activated DCs, we assessed their capacity to stimulate allogeneic lymphocytes. After preincubating DCs with 10 mg/mL Gd, lymphoproliferative capacity was markedly diminished upon maturation as compared with nonpretreated matured DCs (Fig. 4). Pretreatment resulted in a 33% reduction of lymphoproliferative activity. Treatment with Gd alone lead to a 25% increase of median $BrdU^+$ lymphocytes compared with immature DCs. When adding an excess amount of polyclonal antibodies to block this effect, unstimulated DCs retained most of their immature phenotype and lymphoproliferative activity was only increased by 7%. In contrast, pretreatment with Gd in combination with blocking antibodies led to a decrease of lymphoproliferative activity of 20% compared with mature DCs, that equaled a blockage of 1/3 of the aforementioned effect (Fig. 4).

Upon activation with various stimuli, DCs produce IL-12 and IL-10; this cytokine secretion pattern seems to characterize their immunogenic behavior.[24,25] TNF-a-stimulated and IL-1b-stimulated DCs showed a median IL-12 p40 secretion of 3403 pg/mL (range: 2956 to 4302 pg/mL) and a median IL-10 secretion of 103 pg/mL (range: 85 to 142 pg/mL). This pattern changed toward an IL-10-dominant cytokine milieu when Gd was added before

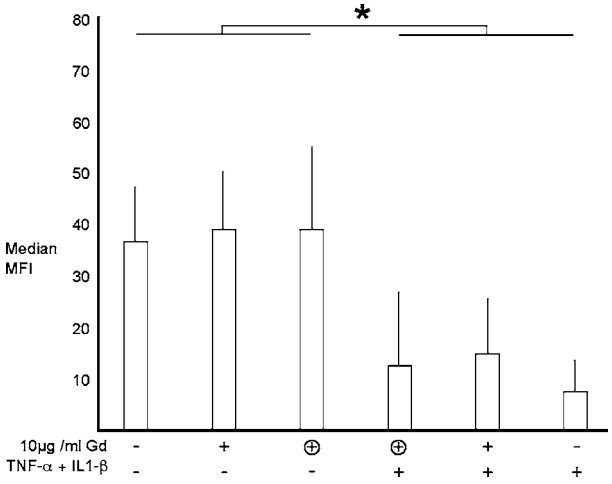

FIGURE 3. Incubation with 10 mg/mL glycodelin (Gd) does not alter endopinocytotic activity compared with matured or unmatured counterparts, respectively. Dendritic cells were incubated with fluorescein isothiocyanate-dextran at 371C for 30 minutes. Mean data from 9 experiments are shown for all nonblocking experiments. Blocking experiments (indicated by a circled +) were performed 3 times. Data show mean fluorescence index (MFI). Error bars indicate SEM. Brackets with an asterisk indicate statistical significance.

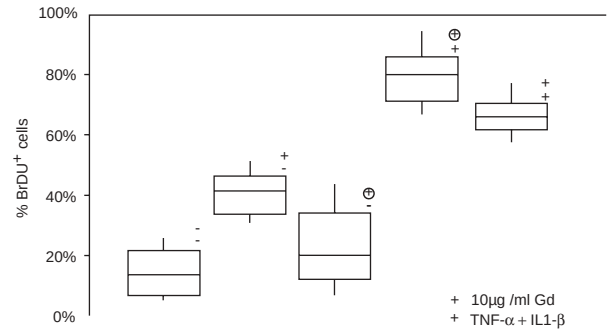

FIGURE 4. Pretreatment with Gd reduces lymphoproliferative activity despite of subsequent maturation. DCs were pretreated with 10 mg/mL Gd for 24 hours and subsequently matured with tumor necrosis factor (TNF)-a (1000 IU/mL) and interleukin (IL)-lb (10 ng/mL). DCs were harvested and cocultured with allogenic peripheral blood monocytes. 5-bromo-2-deoxyuridine (BrdU) incorporation was determined after 72 hour of coculture. The data shown represent cumulative data from 9 experiments for all nonblocking experiments. Blocking experiments (indicated by a circled +) were performed 3 times. Error bars indicate ± SEM. Data were normalized to stimulated DCs. DC indicates dendritic cells; Gd, glycodelin.

maturation. In this case, a median IL-12 p40 secretion of 1956 pg/mL (range: 1689 to 2235 pg/mL) was noted, whereas IL-10 was detected at a median concentration of 357 pg/mL (range: 289 to 453 pg/mL). Low concentrations of IL-10 were detected in both immature DCs [52 pg/mL (range: 26 to 63 pg/mL)] and Gd-treated DCs without subsequent maturation [98 pg/mL (range: 82 to 110 pg/mL)] with only trace IL-12 p40 detectable in these conditions (Fig. 5). Upon incubation with blocking antibodies the IL-10 and IL-12 secretion-pattern of immature DC resembled that of non-Gd preincubated conditions with IL-10 at 118 pg/mL (range: 99 to 154 pg/mL). Matured DCs that were preincubated with Gd, which was blocked with an excess of polyclonal antibodies showed an IL-12 dominant cytokine pattern [3758 pg/mL (range: 2984 to 4689 pg/mL)]

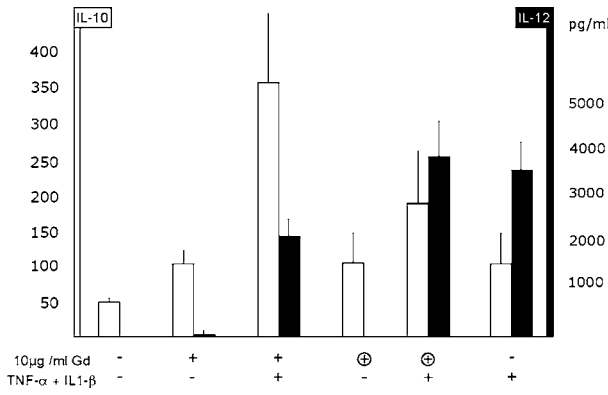

FIGURE 5. Gd-pretreated matured DCs show an IL-10 dominant pattern of cytokine secretion. DCs that were pretreated with 10 mg/mL Gd before maturation with TNF-a (1000 IU/mL) and IL-l b (10 ng/mL) exhibit an $IL\text{-}10^{high}$ $IL\text{-}12^{low}$ cytokine pattern, whereas incubation with Gd without subsequent maturation does not induce IL-10. Maturation of DCs with TNF-a and IL-l b alone resulted in an $IL\text{-}10^{low}$ $IL\text{-}12^{high}$ cytokine pattern. Mean data of 6 independent experiments. Error bars indicate the SEM. DC indicates dendritic cells; Gd, glycodelin.

with a retained IL-10 activity of 186 pg/mL (range: 125 to 251 pg/mL). High Gd concentrations (25 mg/mL) did not change this cytokine pattern, whereas this effect on cytokine secretion was not statistically significant after pretreatment with 5 mg/mL Gd (data not shown).

DISCUSSION

This study shows that monocyte-derived DCs exhibit maturation defects upon incubation with the glycoprotein Gd derived from ascites of ovarian cancer patients.

Isolated Gd from malignant ascites impaired full expression of a mature phenotype as determined by flow cytometric analysis. A fully matured surface marker phenotype ($CD83^+$, $CD86^+$, MHC-II^{high}, and DC-$SIGN^{low}$) was induced upon maturation with a combination of TNF-a and IL-1b, which is in agreement with published data.[6,26] When we preincubated DCs with Gd, however, DCs showed a different reaction to these standard maturation stimuli. Compared with fully matured DC, we saw a markedly reduced expression of costimulatory molecules, which translated into an equally reduced immunostimulatory capacity. MHC-II-dependent antigen presentation, indicated by MHC-II expression, is not impaired by Gd pretreatment, whereas DC-SIGN expression was partially retained compared with mature DCs. DC-SIGN is expressed by immature DCs and constitutes a potent tolerogenic antigen-receptor that binds fucosylated Lac-di-NAc-*N*-glycans with high affinity and contributes to DC-mediated antigen-specific immunosuppression.[27,28] A "semimature" DC pattern has been reported to be characteristic of tolerogenic behavior.[29] Interestingly, we saw a distinct $CD83^+$/DC-$SIGN^+$ population, that is indicative of a tolerogenic phenotype. DC-SIGN is a type II C-type lectin that binds with high affinity distinct carbohydrates such as high mannose structures and fucosylated Lewis blood group antigens (eg, $Lewis^x$); fucosylated Lac-di-NAc-*N*-glycans is bound with the highest affinity.[28,30,31] All of these DC-SIGN binding partners are found on Gd.[32] Gd is the only mammalian glycoprotein that carries the strongest DC-SIGN binding partner, which is otherwise only found on helminths such as *Schistosoma mansonii*.[33] Lac-di-NAc-N-glycans seem to play a central functional role in the immunogenicity of DCs. Schistosoma egg antigen (SEA)-expressing Lac-di-NAc-*N*-glycans inhibit DC maturation and skew subsequent immune responses toward a tolerogenic pattern.[34] Van Liempt et al,[34] (2007) show in their experiments a DC-SIGN-dependent reduction in IL-12 p40 secretion in matured DCs upon incubation with SEA, whereas IL-10 secretion remains high even with low concentrations of SEA. These results are in striking similarity to the effects we see with ovarian cancer-derived Gd, although we were not able to demonstrate DC-SIGN-dependence owing to the scarceness of isolated Gd. One of the decisive factors discriminating immunostimulatory from tolerogenic DCs is their cytokine pattern. DCs secrete IL-12, one of the pivotal cytokines that induce a cytotoxic T-cell immune response, upon various maturation stimuli.[35] In contrast, DCs produce IL-10, the hallmark cytokine involved in tolerogenic immune responses, upon exposure to inhaled antigens, thereby inducing peripheral tolerance and inhibiting misled immune responses such as asthma.[36] Recent studies that examined cancer antigen recognition showed an increased IL-10 production when DCs encounter necrotic cancerous material.[37] We were able to demonstrate that IL-10 was secreted in response to a single cancer-derived glycoprotein, Gd. There is conflicting evidence on whether Gd induces monocytic apoptosis.[38,39] However, IL-10 secretion, which we saw in our experiments, argues against a direct cytopathic effect of Gd on DCs. Moreover, we assessed viability by Trypan dye exclusion, leading toward comparable results at the Gd concentrations used. As a reproductive tract glycoprotein, Gd has been used to stimulate monocytes derived from the uterine deciduas.[40] Gd was able to induce a similar cytokine pattern in our experiments using monocyte-derived DCs from peripheral blood of healthy volunteers compared with decidual monocytes. These decidual monocytes are known to play a central role in tolerogenic mechanisms during pregnancy, when the semiallogenic fetus is accepted by the maternal immune system.[41] Furthermore, spontaneous secretion of tolerogenic cytokines, including IL-10, has been reported for mononuclear cells during the course of pregnancy.[42] Most importantly, DCs were less able to stimulate allogeneic lymphocytes upon encounter of Gd. Peripheral tolerance is one of the central features of an immune response during carcinogenesis and pregnancy.[43,44] In our experiments, we provide morphologic and functional data that a tolerogenic DC state is induced by ovarian cancer-derived Gd, a mechanism that is also employed during early pregnancy. Targeting DCs, this mechanism influences one of the central players of adaptive immunity in a way that supports carcinogenesis. As DCs are employed in various immunotherapy protocols, these data might influence strategies in which whole tumor cell-derived antigen preparations are used to load DCs. In these instances, tolerogenic mechanisms that hamper anticancer immune responses might be induced. Our data contribute to a better understanding of the interactions between tumors and the immune system and may lead to more effective cancer immunotherapy strategies.

ARTICLE PRÉCIS

Ovarian cancer Gd induces a tolerogenic phenotype in dendritic cells and may contribute toward a tumor micromilieu that supports carcinogenesis.

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