母体の血清学的検討から分娩直前のHSV-2の初感染が示唆された。この症例では母体と新生児からそれぞれHSV-2を分離することができた。

この2つのウイルスについてウイルス学的に検討したところ，母体のHSV-2には複数のクローンが含まれていたが新生児のHSV-2にはそのうちの一つのクローンしか感染していないことが判明した[30]。すなわち，複数ある母体のHSV-2のクローンのうちで何らかの選択が行われているらしい。興味深いことに，新生児から分離されたHSV-2は母体のHSV-2に比べ高温でより増殖しやすいことがわかった。大胆な言い方ではあるが，HSV-2による胎内感染の成立には，羊水中のような高温で増殖できるウイルス株であることが関連しているかもしれない。このようにウイルスの側からHSV母子感染について研究した報告は世界的にもみられていない。

XII. 新生児ヘルペスのリスク因子

以上のように，胎内感染による新生児ヘルペスの発症はごく稀であるので，性器ヘルペス合併妊婦の管理の重要な点は，分娩時感染による新生児ヘルペスの発症予防である。

従来，分娩時に性器ヘルペスを合併していると初感染の場合はその50%が，再発では0~2%に新生児ヘルペスを発症するといわれている[31]。母体の性器のHSV感染の病型による新生児ヘルペスの発症率の違いは次のように説明される。まず，病巣の広さと感染ウイルス量が初感染では再発型に比べてはるかに広く，また多量である。感染しているウイルス量は初感染では10^6/0.2 mLであるのに対して再発では10^2～10^3/0.2 mLと1/1,000程度といわれている。さらに，外陰だけでなく子宮頸管からHSVが分離されるのは，初感染では50～70%もの症例にみられるのに対し再発では5%以下と少ない。また，病期についても前者が約3週間に及ぶことがあるが，後者では3～7日と短い。

次に，母体の血清抗体をみると，初感染では急性期では抗体が陰性であり，したがって，児への移行抗体はないのに対して，再発では高いIgG抗体を有しているのでこれが児に移行して受動免疫を賦与することになる。Proberらは，再発型性器ヘルペス合併妊婦では新生児ヘルペスを発症することはほとんどないとして，その理由に母体からの中和抗体の移行によるとしている[32]。後述のように，筆者も再発型40例について妊娠36週以降に母体の中和抗体を測定しているが16倍以上の抗体価を有していた。これらはすべて経腟分娩を行ったが新生児ヘルペスを発症した例はなく，移行抗体が何らかの予防機序に働いているのではないかと考えている。一方，初感染の場合，筆者らの検討ではIgG抗体は7～10病日になって初めて出現し，その抗体価もあまり高くなかった。この点は初感染の性器ヘルペス合併妊婦を取り扱う際に考慮すべき点であろう[33]。

最近Brownらは，母体の免疫状態と新生児ヘルペス発症について詳細に検討している。彼らは4万例以上の全妊婦について分娩時に産道からのHSVの分離と血清抗体の検出を行い，新生児ヘルペス発症のリスクを母体の感染を初発群と再活性化群に分けて検討している[31]。

1 初発群（図6）

ここでいう初発群には，HSVに対する抗体が陰性であった群（初感染）と分離したHSVの型と違う型の抗体が検出された群（HSV-1抗体存在下のHSV-2感染）が含まれる。前者が9例，後者が17例の計26例のうちで外陰に病変がみられた3例では帝切が行われ児に異常はなかったが，外陰に病変のなかった23例のうち17例が経腟分娩を行ったとこ

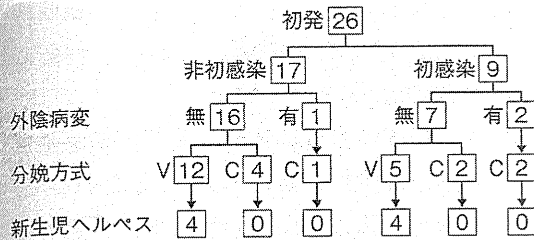

図6　分娩時 HSV 分離陽性妊婦から生まれた児の予後：初発群（文献31より）
注1）初感染　：HSV-1 または HSV-2 分離，抗体陰性
　　非初感染：HSV-2 分離，HSV-1 抗体陽性
　　　　　　　HSV-1 分離，HSV-2 抗体陽性
注2）V：経腟分娩，C：帝王切開

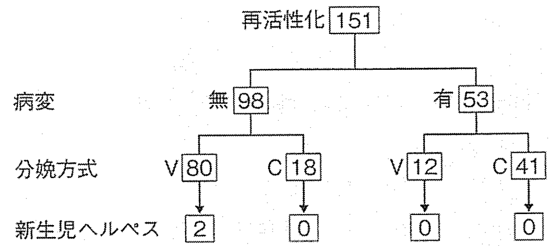

図7　分娩時 HSV 分離陽性妊婦から生まれた児の予後：再活性化群（文献31より）
注1）再活性化：HSV の型と同じ型の抗体をもっている
注2）V：経腟分娩，C：帝王切開

ろ8例に新生児ヘルペスを発症している。詳しく述べると，前者（初感染）では5例中4例（80%），後者では12例中4例（33%）に新生児ヘルペスを発症している。後者でもかなりのリスクがあるが，この感染病態を決めるには感染しているウイルスの型と母体の型特異的抗体を検査する必要がある。

一方，産科的理由で帝王切開を行った6例では新生児ヘルペスは発症していない。この結果は帝王切開は新生児ヘルペスの予防に有効であることを示している。この研究から分娩直前に無症候で HSV に初感染し経腟分娩を行った場合が最もハイリスクであるということになる。ただ，実際には臨床症状がないのでこのような例を発見することは不可能に近い。

2 再活性化群（図7）

ウイルス分離時に HSV の型と同じ型の抗体を有している例（再活性化）が151例あった。そのうち外陰病変のないものが98例あり，その中で経腟分娩を行った80例の中から2例の新生児ヘルペスが生まれている。症状のある場合は帝王切開例が多く，この群から新生児ヘルペスは生まれていないが，興味深いことに症状があっても経腟分娩を行っている12例からも新生児ヘルペスは発症していない。まとめると，再活性化群では産道から HSV が分離されていてしかも経腟分娩を行っても92例中2例（2%）にのみ感染が成立している。おそらく母体からの移行抗体が予防的に働いたのであろう。

さらに，本研究では胎児モニターのため頭皮電極をつけた場合や子宮頸管からの HSV 分離が陽性の場合に新生児ヘルペスのリスクが高くなることなどが示されている。

以上から，母体の感染病態からみた新生児ヘルペス発症のリスク因子は次のようになる。

①妊娠末期の初感染と抗 HSV-1 抗体存在下の HSV-2 感染例
②外陰に病変がある場合
③子宮頸管に HSV が陽性の場合
④母体血中に中和抗体がないか低い場合

外陰に病変がある場合は帝王切開で感染を予防することが可能であるが，無症候の初感染に対する対応が難しい。性器ヘルペス合併妊婦の場合は，子宮頸管の HSV の検出や母体の中和抗体の検出などを分娩前に行えば，ある程度リスクの算定に役立つと思われる。

XIII. 性器ヘルペス合併妊婦の管理

以上を考慮しつつ，筆者らは以下のような管理を行ってきた。

表 8　性器ヘルペスの合併妊娠の管理（発症時）

①病原診断による診断の確定
　ウイルス分離，ウイルス抗原や DNA の検出
②感染病態の決定
　血清抗体の測定（IgG 抗体，IgM 抗体，型特異抗体）
③治　療（表 7 参照）

表 9　性器ヘルペスの合併妊娠の管理（妊娠中）

①外陰・子宮頸管よりウイルス分離または LAMP 法による DNA 検出
　28〜35 週　2 週に 1 回
　36 週〜　　1 週に 1 回
　→陽性の場合はバラシクロビルまたはアシクロビル投与 5 日間
②母体中和抗体ならびに IgG 抗体測定
　34〜38 週

表 10　性器ヘルペス合併妊婦における外陰部と子宮頸管からの HSV の分離

		子宮頸管 HSV 分離		計
		＋	−	
外陰部 HSV 分離	＋	16（19.0%）	68（81%）	84
	−	3（1.3%）	235（98.7%）	238
計		19（5.9%）	303（94.1%）	322

（2008 年 7 月まで）

1 発症時

病原診断と感染病態を明らかにし，治療を行う（表 8）。

2 妊娠中（表 9）

外陰と子宮頸管から HSV の分離培養または LAMP 法による HSV-DNA 検出を行う。おおよそ 28〜35 週まで 2 週に 1 回，36 週以降は 1 週に 1 回行う。検体採取は外陰全体を擦過して採取するが，よく再発する部位からは特に念入りに採取している。無症候で HSV を排出している可能性と子宮頸管からの上行性感染のリスクを考えて，このようにしている。今までの結果をまとめると，外陰から HSV が分離された 84 例のうち 16 例（19%）に子宮頸管からも分離が陽性であった。外陰から HSV が分離されなかった 238 例では 3 例（1.3%）に子宮頸管から分離が陽性であり，外陰に病変がない場合は子宮頸管に HSV が排泄されることはあるが，その頻度はかなり低いようである（表 10）。大切な点は，子宮頸管から分離が陽性であった 19 例のうち 13 例（68%）は初感染であったことで，分娩直前の初感染は経腟分娩による母子感染のリスクが高いことを示している。

筆者らは再発型性器ヘルペスを合併している場合，妊娠末期に母体に中和抗体があればこれが児に移行し児を感染から守ると考えられるので検査を行っている。図 8 は再発性器ヘルペスを合併した妊婦が経腟分娩を行ったが新生児ヘルペスを発症しなかった 40 例について検討したものであるが，HSV-1 による 10 例，HSV-2 による 30 例ともに中和抗体価が 16 倍以上であった。この場合の中和抗体は非補体要求性の抗体である。胎児は補体価が低いので非補体要求性中和抗体による抗体価が重要と考えている。この際，中和抗体を測定するとともにこれが IgG 抗体であることを ELISA 法により確認している。以上のことから，とりあえず中和抗体が 8 倍未満の場合はリスクが高くなるのではないかと考え

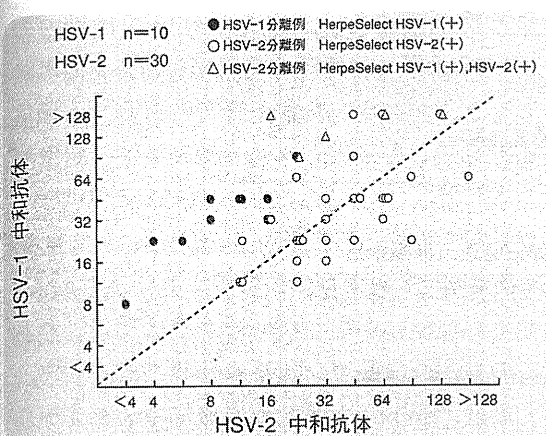

図8 再発型 経腟分娩例 妊娠末期中和抗体価

ているが，今後の検討が必要である。

3 分娩様式の選択（表11）

分娩時に外陰病変があれば帝王切開を行う。外陰病変がなくても初感染では発症より1カ月以内，再発では発症より1週以内はやはり帝王切開を行う。初感染では1カ月以上，再発では1週以上経ていて外陰病変がなければ経腟分娩を行う。初感染の場合，発症から1カ月以上経つとIgGによる中和抗体が産生されるようになると考えられることと，初感染性器ヘルペス合併妊婦でHSVの分離期間は2週間以内が多いが，稀に1カ月間続いた例があったのでこの管理法を考案した。なお，新生児は出生時に目，鼻，口，耳，性器よりHSVの分離を行うとともに臍帯血のIgG抗体，IgM抗体を測定する。新生児は7日以上は入院管理として新生児ヘルペスの早期発見に努める。このような管理方法を行ってきて性器ヘルペス合併妊婦から新生児ヘルペス児は1例も発症していない。ただし，この管理方式はアシクロビルなどの有効な抗ウイルス薬が開発される前に作成したものである。抗ウイルス薬を使用することにより治癒までの期間が著しく短縮されているので，初発の場合でも病変が治癒しウイルス分離が陰性ならば必ずしも1カ月にこだわる必要はなくてもよいであろう。また，再発例についても中和抗体が十分あって抗ウイルス薬を投与して治癒を促進し，ウイルス分離が陰性ならばこの管理方式によることもないであろう。いずれにせよ，抗ウイルス薬使用による管理方式は今後の課題である。

表11 性器ヘルペスの合併妊娠の管理：分娩様式の選択

①分娩時に外陰病変あり・・・・・・・・・・・	帝切
②分娩時に外陰病変なし	
a．初感染・・・・・・発症より1カ月以内	帝切
発症より1カ月以上	経腟
b．再発型または非初感染初発	
・・・・・・発症より1週間以内	帝切
1週以上	経腟

XIV．“再発”性器ヘルペス合併妊婦の管理

妊娠中に性器ヘルペスが再発した妊婦や妊娠する前から再発性器ヘルペスを有している妊婦は，どのように管理するかについてはいろいろな考え方が錯綜している。その理由は，分娩時再発例の経腟分娩による新生児ヘルペスの発症が0〜2%と報告により多少差はあるが，いずれにしてもかなり低率であるので重要視しない考えがある一方で，新生児ヘルペスの悪い予後を考えると2%を重大ととらえる考えがあるからであろう。

オランダでは，分娩時に再発していても経腟分娩を行っているようだし，前述のProberの報告[32]でも再発例では新生児ヘルペスを発症することはまずないと報告しているので，分娩時に再発していても経腟分娩による新生児ヘルペス発症のリスクはかなり低いとみてよいであろう。

英国の2007年のガイドラインをみると，分娩時に再発している場合，産婦に対して新生児ヘルペスの発症のリスクは非常に低いことを告げるようにするべきで，帝王切開を

ルーチンに進めることはないという立場をとっている[34]。このような考えに至る背景には，英国では新生児ヘルペスは6万出生に対して1例と非常に低い頻度であることも関連しているであろう。

しかし，一方では新生児ヘルペスを恐れるあまり病変の有無と関係なくすべて帝王切開分娩にしてしまうこともあるようであるが，筆者らは米国のように[35]病変があれば帝王切開を選択することにしている。病変の有無により分娩様式を選択するという考えに従えば分娩時の再発を抑えれば経腟分娩が可能になる。米国では，性器ヘルペスによる帝王切開がかなり頻繁に行われているようであり，これに対して性器ヘルペスの再発抑制療法を妊娠36週より行って再発を抑制して帝王切開しないで済むようにしようという管理方式が提案された。再発抑制療法とは，妊娠36週からアシクロビル200 mg×4回，またはバラシクロビル500 mg×2回を毎日服用する方法である。1日量としては，非妊婦に行われている再発抑制療法の2倍量が用いられている。最近，Sheffieldらは5つの報告のメタアナリシスを行ってその効果について検証している。それによると，確かに分娩時の再発や再発の前兆は減少し，したがって帝王切開例も有意に減少している[36]。最近，米国のACOGもこの方法を推奨している[37]。今のところはアシクロビルによる新生児の副作用といわれている好中球減少もないという。

しかし，一方で分娩時に再発していて経腟分娩を行っても新生児ヘルペスは発症しないというオランダの経験や，前述のProberの報告[32]，そして筆者らも再発性器ヘルペス例についてほとんどの例で経腟分娩を行ったが新生児ヘルペスの発症例はなかったなどの経験もあり，妊娠36週からの抑制療法を全例について行うことには賛成できない。

また，バラシクロビルを用いて妊娠36週より抑制療法を行っても分娩前7日以内のHSV排泄率と分娩時の発症率は，プラセボ群と有意差がなかったとの報告もある[38]。母体に投与されたアシクロビルは高濃度に胎児や羊水に移行することが知られているので，1カ月にもわたる抑制療法が長期的にみて胎児へ腎機能（アシクロビルは腎より排泄される）や骨髄機能に影響がないのかやはり不安であるので，今後厳重な追跡は必要であろう[39]。

最近，Hollierらが7つの報告のメタアナリシスを行っている。再発抑制療法を行っても新生児ヘルペスの発生を減少させたという証拠はなく，また新生児の長期の副作用に関する検討も十分ではないとしている[40]。

ただ，抑制療法は妊娠末期に子宮頸管からのHSV分離が陽性の例や妊娠10カ月に入ってから頻繁に再発を繰り返す例，そして再発を恐れて精神的に不安定になっている妊婦などには考慮してもよいであろう。

XV. 分娩管理の新しい試み

再発性器ヘルペスを有する妊婦に対する前述の抑制療法は，いつ再発するかわからないので妊娠36週から分娩まで行っているのである。一方，臨床症状から再発を疑って帝王切開したものの後から振り返ると約2/3は誤診していたということも前述のとおりである。これらは，分娩時（入院時）に性器にHSVが感染していることを正確に，そして迅速に知ることのできる方法がないことに起因している。

筆者らは，迅速で感度・特異度ともによい核酸増幅法であるLAMP法が性器ヘルペスの診断に有効であることを確認した。この方法は2時間以内に結果を出せ，培養法とほぼ同じ感度である。この迅速検出法を用いて性器ヘルペス合併妊婦に対して新しい管理方法を案出した。まず入院時に外陰と子宮頸管か

らLAMP法にてHSV-DNAの検出を行い陰性の場合は経腟分娩を行う。陽性の場合は母体の中和抗体が8倍未満か，8倍以上でもウイルス量が多い場合は帝王切開で分娩させるが，ウイルス量が少ない場合で中和抗体が16倍以上あれば経腟分娩を試みる。この16倍以上という抗体価の評価については症例を重ねて検討する必要がある。出生した児については，ただちに目，鼻，口，耳，性器についてLAMP法を施行し，陽性ならばただちに抗ウイルス療法を行う。陰性ならば経過観察とする。産褥3日目にも母体の外陰と子宮頸管と児について検査を行う[41]。

この管理方法はまだ始まったばかりであるが，現在のところ新生児ヘルペス発症例はない。LAMP法が臨床の場で簡単に行うことができれば，再発性器ヘルペスのある妊婦について従来の経験的な管理法や36週からの盲目的な抑制療法を行わなくても母子感染の予防は可能となろう。

●おわりに

現在わが国では，性器ヘルペスを診断するための感度・特異度ともに優れた検査法がないために正確な診断が難しい。さらに，型特異抗体の測定もできないので，性器ヘルペスの感染病態も正しく診断できない。したがって，性器ヘルペス合併妊娠における精度の高い管理法はできないのが現状である。これらの方法が保険で使用できるようになることを切望している。

そして，最後に最も重要なことは，新生児ヘルペスを出生した母体の約70%は無症候であったということである。いかにこのようなリスクのある例を抽出して，HSVの母子感染を予防するかが今後の大きな課題である。一つの方法として，妊婦とその夫についてHSVの（型特異）抗体を検出しておいて，もし妊婦が抗体陰性で夫が陽性の場合，妊娠中期以降の性行為を禁止するかコンドームを使用させてHSVの感染を予防するなどの試みが提案されている[42]。ただ，全妊婦にスクリーニングを行うこの試みは費用対効果を考えると新生児ヘルペスの少ない地域では必ずしも適切な方策とはいえない[43]。いずれにせよ，新生児ヘルペス発症のハイリスク妊婦が特定できるような方策の確立が望まれる。

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ORIGINAL ARTICLE

Subsequent risks for cervical precancer and cancer in women with low-grade squamous intraepithelial lesions unconfirmed by colposcopy-directed biopsy: results from a multicenter, prospective, cohort study

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Abstract
Objective To investigate the natural course of low-grade squamous intraepithelial lesions (LSILs) that cannot be histologically confirmed by colposcopy-directed biopsy.
Methods In a multicenter, prospective, cohort study of Japanese women with LSILs, we analyzed the follow-up data from 64 women who had a negative biopsy result at the initial colposcopy (biopsy-negative LSIL) in comparison with those from 479 women who had a histologic diagnosis of cervical intraepithelial neoplasia grade 1 (LSIL/CIN1). Patients were monitored by cytology and colposcopy every 4 months for a mean follow-up period of 39.0 months, with cytologic regression defined as two consecutive negative smears and normal colposcopy.
Results In women with biopsy-negative LSILs, there were no cases of CIN3 or worse (CIN3+) diagnosed within 2 years; the difference in the 2-year risk of CIN3+ between the two groups was marginally significant (0 vs. 5.5%; $P = 0.07$). The cumulative probability of cytologic regression within 12 months was much higher in the biopsy-

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negative LSIL group (71.2 vs. 48.6%; $P = 0.0001$). The percentage of women positive for high-risk human papillomaviruses (hrHPVs) was significantly lower in the biopsy-negative LSIL group than in the LSIL/CIN1 group (62.1 vs. 78.4%; $P = 0.01$); however, the 12-month regression rate of biopsy-negative LSIL was similar between hrHPV-positive and -negative women (67.3 vs. 74.4%, $P = 0.73$).

Conclusion In women with biopsy-negative LSILs, the risk of CIN3+ diagnosed within 2 years was low; furthermore, approximately 70% underwent cytologic regression within 12 months, regardless of HPV testing results. Biopsy-negative LSILs may represent regressing lesions rather than lesions missed by colposcopy.

Keywords Low-grade squamous intraepithelial lesion · Colposcopy · Human papillomavirus · Cervical intraepithelial neoplasia

Introduction

In the Bethesda System for cytologic reporting, a low-grade squamous intraepithelial lesion (LSIL) represents mild cervical abnormalities, including cellular changes associated with human papillomavirus (HPV) infection and cervical intraepithelial lesion grade 1 (CIN1) [1]. However, approximately 15–20% of women with a cytologic interpretation of LSIL have a grade 2 (CIN2) or grade 3 (CIN3) cervical intraepithelial lesion, which are immediately treated with cervical ablation, loop electrosurgical excision procedure (LEEP) or cone biopsy [2, 3]. Therefore, women with LSILs usually undergo a colposcopy-directed biopsy for histologic evaluation of cervical abnormalities. Of the women with LSIL cytology, 40–60% are found to have a histologic diagnosis of CIN1 at the initial colposcopy, while 15–30% have a negative biopsy result [2, 3]. According to the 2006 American Society for Colposcopy and Cervical Pathology consensus management guidelines [4], the follow-up strategy for women with a negative biopsy result is identical to that of women with CIN1; that is, both groups are followed with either repeated cytology at 6 and 12 months or HPV testing at 12 months. However, the natural course of LSILs that cannot be histologically diagnosed by colposcopy-directed biopsy has not been well documented.

The Japan HPV and Cervical Cancer (JHACC) cohort study was designed to identify determinants of regression and progression of low-grade cervical abnormalities [5, 6]. In the primary analysis, we used only the follow-up data from 570 women with cytologic LSIL and histologically confirmed CIN1 or CIN2 lesions, and demonstrated HPV type-specific risks of LSIL persistence and progression [5]. In the present study, we analyzed the follow-up data of 64 women with biopsy-negative LSIL who were excluded from the main analysis cohort.

Methods

Study design

This study represents a secondary analysis of data from the prospective non-intervention cohort study conducted by the JHACC study group for identifying determinants of LSIL/CIN regression and progression. Details of the design, methods, and primary results have been provided in more detail elsewhere [5, 6]. Briefly, 905 women with mildly abnormal cytology were recruited from nine hospitals that performed conventional Pap smears, colposcopy and cervical biopsies. The inclusion criteria of this secondary analysis were: evident LSIL cytology; histologic diagnosis of CIN1 or less at initial colposcopy and biopsy; age 18–54 years; first detection of cervical abnormality; and a sufficient number (two or more) of follow-up visits. Women entered the study voluntarily after giving their signed informed consent. Cervical smears were classified according to the Bethesda System [1]. At the time of study entry, two (or more) small cervical specimens were taken by colposcopy-directed punch biopsy and stained with hematoxylin and eosin (H&E). A histologic diagnosis was determined based on the World Health Organization (WHO) classification system. Two cytopathologists (Y.H. and Masafumi Tsuzuku) and two pathologists (R.F. and T.K.) reviewed all cytologic and histologic specimens collected at the time of entry. Patients were tested for cervical HPV DNA, serum IgG antibodies to cytomegalovirus (CMV), *Chlamydia trachomatis*, and herpes simplex virus type 2 (HSV2) at the time of entry. The researchers who performed the assays were blinded to the clinical data collected from the study subjects. Information regarding smoking and sexual behavior was obtained from a self-administered questionnaire. Patients were routinely followed at 3- to 4-month intervals and received cytologic and colposcopic examinations at each visit. To avoid interference from the biopsy procedure on the natural course of the disease, a cervical biopsy was performed during the follow-up period only when Pap smears and colposcopic findings were suggestive of the presence of CIN3 or worse (CIN3+). A cytology result of HSIL triggered colposcopy-guided biopsy during follow-up examinations. The two cytopathologists and the two pathologists reviewed all cytologic and histologic specimens collected for the diagnosis of CIN3+. We chose an end-point of CIN3 or cancer rather than CIN2 or higher because CIN2 likely represents a heterogenous collection of cervical abnormalities [7, 8], only some of which progress to CIN3 [5, 9]. In this analysis, we defined regression as normal colposcopy results and at least two consecutive negative Pap smears. Persistent lesions were defined as lesions that did not regress or were diagnosed with CIN3+ during the follow-up period.

Overall, the study subjects consisted of 554 women who had a negative biopsy result (biopsy-negative LSIL; $n = 64$) or a histologic diagnosis of CIN1 (LSIL/CIN1; $n = 491$) at the initial colposcopy for LSIL cytology. Unfortunately, data from cervical samples, blood samples, or questionnaires were not available in all 554 study subjects. Cervical HPV data were not available in 21 women because of insufficient samples, while data on serum antibodies to sexually transmitted agents were lacking in 23 women. In addition, 54 women gave no responses to a self-administrated questionnaire. The study protocol was approved by the ethical and research review boards of the participating institutions.

HPV genotyping

We detected HPV DNA in cervical samples by polymerase chain reaction (PCR)-based methodology, as previously described [10]. In brief, exfoliated cells from the ectocervix and endocervix were collected in a tube containing 1 ml of phosphate-buffered saline (PBS) and stored at −30°C until DNA extraction. Total cellular DNA was extracted from cervical samples by a standard sodium dodecyl sulfate (SDS)–proteinase K procedure. HPV DNA was PCR amplified by using consensus primers (L1C1/L1C2 + L1C2M) for the HPV L1 region. A reaction mixture without template DNA was included in every set of PCR runs as a negative control. Primers for a fragment of the β-actin gene were also used as a control to rule out false-negative results for samples in which HPV DNA was not detected. HPV types were identified by an analysis of restriction fragment length polymorphism (RFLP), which has been shown to identify at least 26 types of genital HPVs [11].

IgG antibody against sexually transmitted agents

The level of IgG antibodies to *Chlamydia trachomatis* and HSV2 was determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits: *Chlamydia trachomatis* (HITAZYME; Hitachi Chemical, Tokyo, Japan) and HSV2 (HerpeSelect 2 ELISA IgG; Focus Diagnostics, Cypress, CA, USA). The serologic assay for *Chlamydia trachomatis* utilizes purified EB outer-membrane proteins of the *Chlamydia trachomatis* L2 strain as antigens and does not detect antibody to *Chlamydia pneumoniae* [12]. These serologic assays were performed at a clinical testing laboratory (SRL, Tokyo, Japan).

Statistical analysis

All time-to-event analyses were based on the actual date of the visits. For regression or progression, time to event was measured from the date of the index visit (i.e., the first instance of an abnormal cytology result) to the date of the visit at which cytologic transition to normal occurred or CIN3+ was first detected. Women whose lesions persisted or who dropped out of the study were censored at their last recorded return visit dates. Subjects who had only one negative colposcopy/cytology result before loss to follow-up were censored at the last date of positive Pap tests. Subjects who were biopsied were censored at the time of their biopsy, regardless of the biopsy results, to reduce the potential for interference by the biopsy procedure on estimates of time of regression. Cumulative probability of LSIL regression or progression was estimated by using the Kaplan–Meier method and compared with a log-rank test. All analyses were carried out using the JMP 7.0J (SAS Institute, Cary, NC, USA) statistics packages. Two-sided P values were calculated throughout and considered to be significant at less than 0.05.

Results

We analyzed the follow-up data from a total of 554 women with LSIL cytology who had a negative biopsy result (biopsy-negative LSIL; $n = 64$) or a histologic diagnosis of CIN1 (LSIL/CIN1; $n = 491$) at the initial colposcopy. Distributions of baseline characteristics between these two groups are presented in Table 1. The women with biopsy-negative LSILs were older than the women with LSIL/CIN1 (mean age ± SD 38.8 ± 9.2 vs. 36.2 ± 7.7 years); however, the difference in the age distribution between the two groups was only marginally significant ($P = 0.07$). Cervical HPV infections were found in 75.0% of women with biopsy-negative LSILs and in 84.6% of women with LSIL/CIN1 results and the difference was statistically significant ($P = 0.02$). The percentage of women positive for high-risk human papillomaviruses (hrHPVs) was also significantly lower in the biopsy-negative LSIL group than in the LSIL/CIN1 group (62.1 vs. 78.4%; $P = 0.01$). The percentage of women who had smoked was lower in the biopsy-negative LSIL group (32.6 vs. 48.7%), but the difference was only marginally significant ($P = 0.07$). The number of lifetime sexual partners was significantly greater among women with LSIL/CIN1 than among women with biopsy-negative LSILs ($P = 0.001$). The age at first sexual intercourse was also lower among women with LSIL/CIN1 compared to women with biopsy-negative LSILs, although the difference was only marginally significant ($P = 0.06$). Women with LSIL/CIN1 were likely to have a higher IgG antibody titer against *Chlamydia trachomatis* than women with biopsy-negative LSILs; however, the difference was not significant ($P = 0.25$). The IgG reactivity to HSV2 was similar between the two groups ($P = 0.82$). At least two

Table 1 Characteristics of the study subjects

	Cytology and histology		P values[†]
	Biopsy-negative LSIL (n = 64)[a]	LSIL/CIN1 (n = 479)	
Age (years)			
Mean (SD)	38.8 (9.2)	36.2 (7.7)	
18–29	11 (17.2%)	95 (19.8%)	0.07
30–39	21 (32.8%)	215 (44.9%)	
40+	32 (50.0%)	169 (35.3%)	
HPV typing			
Positive for high-risk types[b]	36 (62.1%)	359 (78.0)	0.01
Negative for high-risk types	22 (37.9%)	101 (22.0%)	
Positive for any HPV	48 (77.4%)	405 (88.0%)	0.02
Negative for any HPV	14 (22.6%)	55 (12.0%)	
Smoking			
Never smokers	37 (63.8%)	222 (51.3%)	0.07
Smokers	21 (36.2%)	211 (48.7%)	
Current smokers	16 (27.6%)	143 (33.0%)	
Former smokers	5 (8.6%)	68 (15.7%)	
Number of lifetime sexual partners			
1	23 (39.6%)	79 (18.1%)	0.001
2–3	13 (22.4%)	129 (29.5%)	
4+	22 (37.9%)	229 (52.4%)	
Age at first sexual intercourse (years)			
≤20	12 (20.3%)	147 (34.2%)	0.06
21–23	26 (44.1%)	179 (41.6%)	
≥24	21 (35.6%)	104 (24.2%)	
IgG antibodies to *Chlamydia trachomatis*			
Low	27 (45.0%)	166 (36.1%)	0.25
Mid	20 (33.3%)	150 (32.6%)	
High	13 (21.7%)	144 (31.3%)	
IgG antibodies to HSV2			
Low	23 (38.3%)	158 (34.3%)	0.82
Mid	19 (31.6%)	150 (32.6%)	
High	18 (30.0%)	152 (33.0%)	

[†] These P value were calculated by the χ^2 test

[a] Biopsy-negative LSIL denotes women with LSILs that had a negative biopsy result at the initial colposcopy

[b] HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 were classified into high-risk HPV types

biopsies were taken at the initial colposcopy and there was no difference in the number of biopsies between the two groups.

Patients were monitored by cytologic and colposcopic testing at intervals of 3–4 months. Among women with biopsy-negative LSILs, no case was diagnosed with CIN3+ within 2 years; the difference in the cumulative risk of CIN3+ diagnosed within the next 2 years between the two groups was marginally significant (0 vs. 5.5%; $P = 0.07$ by log-rank test; Fig. 1a). In women with biopsy-negative LSILs, the majority of cytologic regression occurred within 12 months. The cumulative probability of cytologic regressions within 12 months was much higher in women with biopsy-negative LSILs than in women with LSIL/CIN1 (71.2 vs. 48.6%; $P = 0.0001$; Fig. 1b). The 2-year rate of cytologic regression was also significantly different between the two groups (75.1 vs. 64.0%; $P = 0.003$). Cytologic regression occurred more quickly in women with biopsy-negative LSILs than in women with LSIL/CIN1 (median time to regression: 6.3 vs. 12.4 months). In the women with biopsy-negative LSILs, the 12-month cumulative probability of cytologic regression was similar between hrHPV-positive and -negative women (67.3 vs. 74.4%; $P = 0.74$); median time to regression was also similar between hrHPV-positive and -negative women (5.4 vs. 7.7 months; $P = 0.45$; Fig. 2a). In women with LSIL/CIN1, however, detection of hrHPVs significantly influenced the 12-month rate of cytologic regression (hrHPV-positive [45.2%] vs. hrHPV-negative [62.6%]; $P = 0.006$; Fig. 2b).

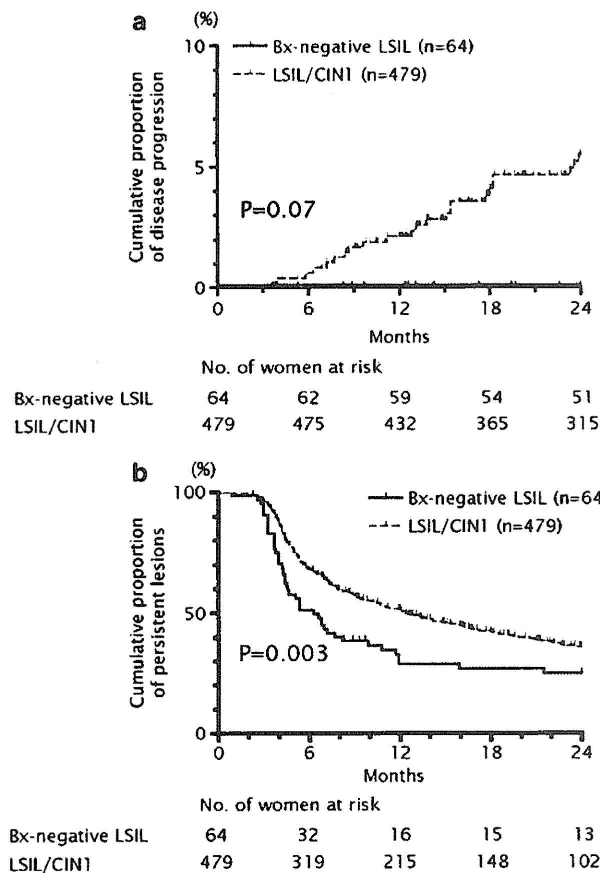

Fig. 1 Cumulative probabilities of CIN3+ diagnosis and cytologic regression within 2 years. A Kaplan–Meier plot was used to estimate the cumulative probabilities of CIN3+ diagnosis (**a**) and cytologic regression (**b**) within 2 years among women with biopsy-negative LSILs (*solid line*) or LSIL/CIN1 (*dashed line*). *P* values were calculated by the log-rank test

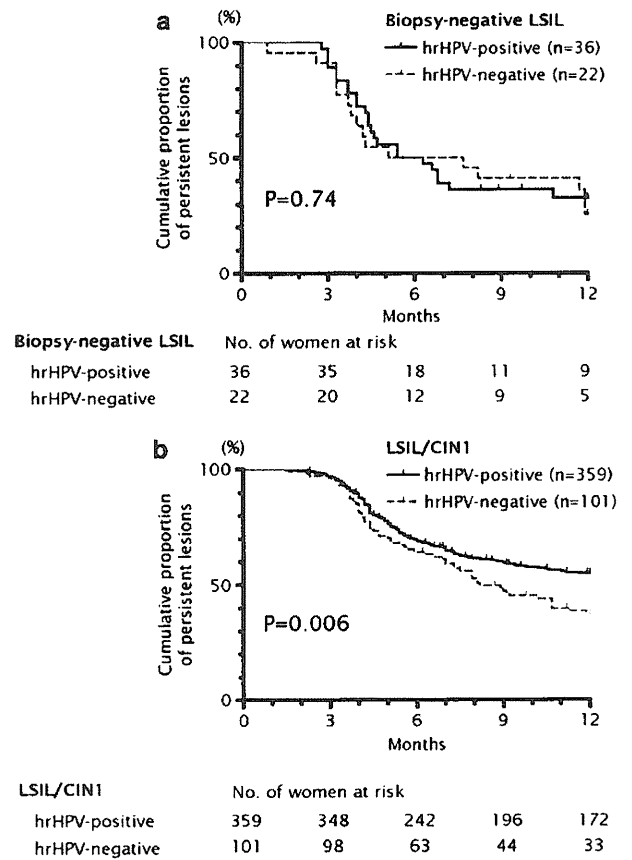

Fig. 2 Cumulative probabilities of cytologic regression within 12 months according to detection of hrHPVs. A Kaplan–Meier plot was used to estimate the cumulative probabilities of cytologic regression within 12 months among women with biopsy-negative LSILs (**a**) or LSIL/CIN1 (**b**) according to hrHPV detection. *P* values were calculated by the log-rank test

Discussion

Colposcopy-directed biopsies are recommended for women with LSIL cytology, primarily to exclude a high-grade lesion. Although approximately 15–30% of those women have a negative biopsy result [2, 3], they are routinely subjected to follow-up because of uncertainty about the risk of precancerous lesions missed by a colposcopic biopsy. In the present study, women with a biopsy-negative LSIL (i.e., "unconfirmed" LSIL) were at substantially low risk of CIN3 or cancer diagnosed within the following 2 years. The women with biopsy-negative LSILs were also significantly more likely to have cytologic regression than women with LSILs underlying CIN1. Some cases of biopsy-negative LSIL may be based on false-positive cytology because the percentage of women negative for any HPV was significantly higher in the biopsy-negative LSIL group than in the LSIL/CIN1 group. Additionally or alternatively, biopsy-negative LSILs may represent currently regressing lesions. This may be supported partially by the higher percentages of women in the biopsy-negative LSIL group who did not have cervical cancer risk factors, such as detection of hrHPVs, smoking, higher sexual activity and infections with *Chlamydia trachomatis* [13–16]. Several studies have reported that LSIL is more likely to regress to normal cytology among hrHPV-negative women or women who never smoked [5, 6, 17]. Interestingly, the 12-month regression rate of biopsy-negative LSIL was high, even among hrHPV-positive women. Low-grade lesions currently regressing to normal cytology may be difficult to confirm by colposcopy-guided biopsies because of the small lesion size, lower-grade colposcopic impression and/or weak pathologic findings.

Data on the natural course of biopsy-negative LSILs are limited. Pretorius et al. [18] reported that the subsequent risk of CIN3+ among women with histologically unconfirmed atypical squamous cells of undetermined significance (ASC-US) or LSIL cytology was low (1.8%). This

result was consistent with our observation; however, it was based on retrospective analyses of previous data including ASC-US cytology. In the ALTS (ASCUS-LSIL Triage Study) report [2], the risk of CIN3+ diagnosed within 2 years after unconfirmed LSIL was higher compared with the present study (6.1 vs. 0%). The difference between our results and the ALTS data may be explained by the difference in study design between the two studies. In the ALTS study, all women had an exit colposcopy and biopsy at 2 years after the semiannual follow-up by repeated cytology. Although our study subjects received both cytologic and colposcopic examinations at each visit at 3- to 4-month intervals, we did not routinely perform a colposcopic biopsy 2 years later. This may have resulted in an underestimation of the 2-year risk for CIN3+ in our study. Additionally, the sensitivity of the enrollment colposcopy may have affected the results from these two prospective studies. Recent studies have showed that initial colposcopy-directed biopsy are not as sensitive as we had previously assumed [19]. Thus, at least two directed biopsies, random biopsy or endocervical curettage are recommend to increase the sensitivity of the initial colposcopy [20–22]. In the ALTS study, 77.6% of women had null or only one biopsy at enrollment colposcopy [20]. By contrast, two (or more) biopsies were taken at entry in our study subjects. The number of biopsies may have increased the risk of misclassification errors of cervical lesions at enrollment. Although central pathologic review systems were employed in both studies, the limitation of histopathologic diagnosis (i.e., poor reproducibility in CIN grading) may also have affected disease classification at enrollment and during follow-up [7, 8, 23].

The current US guidelines advise that women with LSIL cytology and a histologic diagnosis of CIN1 or less should be followed with repeated cytology at 6 and 12 months or, alternatively, hrHPV testing at 12 months [4]. Our data also confirmed that these management strategies are sufficiently safe. A previous study reported that there was no significant difference in the subsequent risk of CIN2/3 between women with no disease documented by initial colposcopy-directed biopsy and women with histologically confirmed CIN1 [24]. However, the study was based on retrospective analyses, which was limited by the small sample size (negative biopsy $n = 43$; CIN1 $n = 30$) and included women with various cytologic abnormality profiles. In the present study, the risk of CIN3+ diagnosed within the following 2 years and the likelihood of LSIL regression were obviously different between women with biopsy-negative LSILs and women with LSIL/CIN1. The 2-year follow-up in ALTS of women with CIN1 or less has indicated that the subsequent risk of CIN2 or higher varies little with respect to the findings at the initial colposcopy [2]. However, when the analysis was confined to the risk of CIN3 or higher among women with LSILs, there was a marginal tendency for a higher risk of subsequent CIN3 that was associated with CIN1 compared with <CIN1 (10.5 vs. 6.1%). Based on these observations, the follow-up strategy for women with biopsy-negative LSILs may be better differentiated from that for women with LSIL/CIN1 results in terms of quality-of-life and cost. Our data suggest that follow-up by repeated cytology at 12 months may be appropriate for women with biopsy-negative LSIL when two or more colposcopy-directed biopsies are taken at the initial colposcopy.

In conclusion, the risk of CIN3+ diagnosed within 2 years was low in women with biopsy-negative LSILs; furthermore, approximately 70% showed cytologic regression within 12 months, regardless of HPV testing results. Our data suggest that biopsy-negative LSILs may represent false-positive cytology or currently regressing lesions rather than lesions missed by colposcopy. However, the sample size of the present study was small; thus, to confirm our results, further prospective studies with larger sample sizes will be needed.

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Conflict of interest We declare that we have no conflict of interest relevant to this article. The supporting organization played no role in the design and implementation of the study; the collection, management, analysis, and interpretation of the data; and the preparation, review, or approval of the manuscript.

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ORIGINAL ARTICLE

A Possible Coagulation-Independent Mechanism for Pregnancy Loss Involving $\beta_2$glycoprotein 1-Dependent Antiphospholipid Antibodies and CD1d

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Keywords
Antibody cross-linking, CD1d, phosphatidylserine, recurrent pregnancy loss, $\beta_2$glycoprotein1 (β2GP1) antibody

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Problem
$\beta_2$glycoprotein1 (β_2GP1)-dependent antiphospholipid antibodies (aPL) increase the risk for recurrent pregnancy loss. We address whether anti-β_2GP1 antibodies can interact with phosphatidylserine (PS)-bearing CD1d on trophoblast cells and induce local inflammation.

Methods
CD1d-bearing choriocarcinoma cells were used in flow cytometry and immunoprecipitation experiments. CD1d-mediated cytokine induction was assessed using antibody cross-linking. Cytokine production during co-culture of decidual lymphocytes with CD1d-bearing cells was also examined.

Results
Trophoblast surface-expressed CD1d forms a complex with PS-bound β_2GP1. Anti-β_2GP1 mAb cross-linking causes IL12p70 release from CD1d-bearing cells. IL12p70 release from CD1d-bearing trophoblast cells was also induced during co-culture with human decidual lymphocytes. The addition of anti-β2GP1 mAb to co-cultures resulted in a three-fold increase in IL12p70 secretion. IFNγ secretion from decidual lymphocytes was also induced during co-culture with anti-β2GP1 mAbs.

Conclusions
β_2GP1-dependent IL12 release from CD1d-bearing trophoblast in the presence of aPL may link the antiphospholipid syndrome to pregnancy loss via an inflammatory mechanism.

Introduction

Antiphospholipid syndrome (APS) is characterized by the production of autoantibodies against negatively charged membrane phospholipid-dependent antigens, including cardiolipin and phosphatidylserine. APS is clinically associated with thrombocytopenia, thrombosis, and pregnancy loss.[1,2] The major target antigen for antiphospholipid antibodies (aPL) has recently been identified as β_2GP1. β_2GP1 molecule is present in the peripheral blood and can act as an inhibitor of the intrinsic coagulation cascade,[3] platelet aggregation, and the prothrombinase activity of activated platelets *in vitro*.[4,5] The role of anti-β2GP1 antibodies in APS-related pregnancy loss may involve interference with the activity of β2GP1 bound to phospholipids on

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activated platelets and the induction of coagulation in the placenta.[6,7] This would suggest that anti-β2GP1 antibody-mediated pregnancy loss occurs after the establishment of blood flow through the placental vasculature (i.e. 9–10 weeks of gestation) and would exclude those anti-β2GP1 antibody-mediated losses known to occur during the first trimester. Examination of first-trimester decidua and trophoblast tissues from pregnancies complicated by the APS, however, has revealed little evidence of specific thrombotic placental pathology.[8] Pure thrombotic events cannot account for all the histopathologic findings in placentae from women with APS.[9]

CD1d is an MHC I-like molecule that presents self- or microbe-derived glycolipid rather than peptide antigens.[10] Its immune effectors are typically natural killer T (NKT) cells.[10] CD1d presents lipid antigens including bacterial and self-lipid. Phosphatidylserine (PS), phosphatidylethanolamin (PE) and phosphatidylinositol (PI) have been known to be presented by CD1d.[11] In humans, a specific subset of NKT cells expresses an invariant Vα24Jα18/Vβ11 T-cell receptor (iTCR) and can recognize CD1d on the surface of antigen-presenting cells (APCs) through this receptor. The activation of invariant NKT (iNKT) cells is antigen dependent, but the antigen itself can be derived from an invading microbe or possibly the host itself.[12] Recognition of CD1d by iNKT cells causes rapid release of IL4 and IFN-γ from the iNKT cell and thereby modulates the Th1/Th2 polarization of adaptive immune cells.[10] The function of CD1d can be addressed experimentally using monoclonal antibody (mAb) cross-linking of cell surface-expressed CD1d. CD1d cross-linking using an anti-CD1d mAb (51.1) induces tyrosine phosphorylation in the CD1d cytoplasmic tail, intracellular signaling through NF-κB and autocrine cytokine release from CD1d-bearing cells.[13,14] As CD1a does not have a tyrosine in its cytoplasmic tail, this cascade does not occur upon cross-linking of CD1a or chimeric molecules that include the CD1a cytoplasmic tail (e.g. CD1d/a, a chimera composed of the extracellular and transmembrane domains of CD1d and the cytoplamic tail of CD1a).[13]

Normal placental extravillous trophoblast cells (EVT) express the MHC class I-like molecule, CD1d, when analyzed by immunohistochemistry.[15] We have reported that CD1d is expressed on the surface of early extravillous trophoblast cells using transient primary cultures of cells isolated from normal placentae during early pregnancy.[16] We have also demonstrated that CD1d expression is regulated *in vivo* in a trophoblast differentiation-dependent manner. CD1d is strongly expressed in EVT located proximally in trophoblast cell columns.[17] Here, the proximity of EVT and iNKT cells would allow interaction of CD1d and its ligand during early placental formation, a process that requires a local pro-inflammatory milieu to promote invasion of EVT into the maternal decidua.[17] CD1d expression in EVT decreases in the distal trophoblast cell columns that invade into the decidua and differentiate into interstitial or endovascular trophoblast cells.[17] These distal EVT cells are closely opposed to a great number of decidual stromal cells and infiltrating decidual immune cells (including decidual iNKT cells). Both NKT cells in the decidua and the peripheral blood had an ability to rapidly produce cytokine associated with Th1 and Th2.[18] It has reported that the percentages of IL4 and IFNγ producing NKT cells were significantly increased in the decidua compared with the peripheral blood in pregnancy.[18] As massive activation of iNKT cells induces pregnancy loss [19,20] the activation of decidual iNKT cells must be tightly regulated. Decreased CD1d expression in distal cell columns may control global activation of iNKT cells. More detailed descriptions of human placental CD1d regulation and expression patterns have been difficult to obtain using stable culture system.

We hypothesized that some APS-associated pregnancy loss may be mediated by anti-β_2GP1 antibody-dependent inflammation at the maternal–fetal interface. We further implicate trophoblast-expressed CD1d molecules as integral to induction of this inflammation. In this work, we demonstrate that β_2GP1-dependent aPL promote IL12 release from CD1d-bearing extravillous trophoblast cell lines, and subsequent IFNγ production by decidual lymphocytes. This, in turn, could cause further activation of inflammatory cells, damage to trophoblast cells, and pregnancy loss.

Materials and methods

Cell Lines

Although we have reported a transient primary culture system for human trophoblast cells isolated from normal placenta during early pregnancy,[21] these cells are difficult to maintain in culture. We therefore used the well-described human trophoblast cell line, Jeg3, for the present studies. While Jeg3 cells are often used *in vitro* to represent extravillous

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cytotrophoblast cells, they do not endogenously express CD1d, which is expressed only in proximal cell column EVT *in vivo*.[16] We therefore transduced CD1 genes into Jeg3 cells using a retroviral vector, pSR-neo, expressing CD1d or a CD1d/a chimeric molecule (kind gifts from Dr. R. S. Blumberg, Harvard Medical School, Boston, MA, USA).[13] Transduced cells were cultured in RPMI1640 (Invitrogen Corporations, Carlsbad, CA, USA) media supplemented with 10% FBS (Invitrogen Corporations) and 500 μg/mL of geneticin/neomycin (Invitrogen Corporations). Neomycin-resistant stable cell lines were established and named Jeg3/CD1d or Jeg3/CD1d/a cells. The chimeric molecule expressed in Jeg3/CD1d/a cells consisted of the extracellular and transmembrane components of CD1d fused to the cytoplasmic tail of CD1a.

Isolation and Culture of Human Decidual Lymphocytes

Human decidual iNKT cells were purified from human decidual tissue isolated after elective termination of uncomplicated first trimester pregnancies under the approval of the Ethical Committee of the Medical Faculty, University of Tokyo. Purified decidual iNKT cells were cultured as previously described.[22] Briefly, minced decidual tissues were digested with 10% collagenase (20 μL) and 20 U/mL DNase typeI (Invitrogen Corporations) in 10 mL RPMI 1640 containing 10%FBS and passed through mesh (100 μm pore size; Invitrogen Corporations). Cells were resuspended in PBS, layered over 5 mL of Ficoll (Sigma-Aldrich Inc., St. Louis, MO, USA) and centrifuged at $800 \times g$ for 20 min at room temperature. Cells at the interface between the PBS and Ficoll layers were collected, resuspended in RPMI1640 containing 10% FBS and 100 ng/mL of αGalCer and cultured at 37°C in 5% CO_2. An aliquot of the isolated lymphocytes was incubated with anti-Vα24-RPE or anti-Vβ11-FITC Abs (1 μg/mL) and analyzed by flow cytometry to confirm the presence of Vα24Vβ11 iNKT cells (data not shown).

In co-culture experiments, 10^5 αGalCer-stimulated decidual lymphocytes were incubated with 10^5 Jeg, Jeg/CD1d, or Jeg/CD1d/a cells at 37°C in 5% CO_2 for the times indicated. For experiments requiring exposure to anti-β2GP1 antibodies, anti-β2GP1 antibody or isotype-control antibodies were added to culture supernatants at a concentration of 10 μg/mL.

Flow Cytometry

Jeg3 cells were detached from culture plates with 0.05% EDTA in PBS and incubated with anti-CD1d (NOR3.2 Abcam Inc., Cambridge, MA, USA), anti-β_2GP1 (CHEMICON International, Temecula, CA, USA) or anti-PS (upstate (Millipore), Billerica, MA, USA) mAbs (1 μg/mL) in PBS for 30 min. For indirect staining experiments, cells were incubated with RPE anti-mouse IgG (Dako Cytomation, Glostrup, Denmark) for 30 min. Controls were exposed to an isotype-matched irrelevant mAb (1 μg/mL; Dako Cytomation). After washing, cells were subjected to flow cytometric analysis for RPE.

Immunoprecipitation and Western Immunoblotting

Cells were cultured in 10-cm plates in RPMI and used at near confluence. Equivalent aliquots of cell lysates were incubated overnight at 4°C with 10 μg/mL of anti-CD1d mAb or anti-AnnexinV Ab (BioVision, Mountain View, CA, USA) and 60 μL of Protein-G-Sepharose (GE Healthcare Bio-science, Piscataway, NJ, USA). As PS cannot be detected by Western blotting, these immunoprecipitants were immunoblotted with a mAb to Annexin V, a protein known to bind tightly to PS.[23] Precipitated proteins were separated across 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. β_2GP1 levels were determined by Western immunoblotting (ECL advance Western blotting detection kit; GE Healthcare Bio-science, Piscataway, NJ, USA). The expression of β_2GP1 was detected using an anti-β_2GP1 mAb labeled with HRP (Peroxidase Labeling Kit; Roche Diagnostics, Lewes, UK).

CD1d Cross-Linking

Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells were cultured in 12-well plates in RPMI and used at 80% confluence. Monolayer cells were exposed to ten [10] μg/ml of anti-CD1d (51.1; eBioscience, San Diego, CA, USA), anti-β_2GP1 (CHEMICON International) or isotype control (DakoCytomation) mAbs and incubated for 1 hr at 37°C. This anti-β_2GP1 antibody is non-functional and binds to two molecules of β_2GP1. Cells were washed with PBS and exposed to 10 μg/mL goat anti-mouse Ig antibody (CHEMICON International) as a cross-linker for 30 min at 37°C. Cells used in the anti-β_2GP1 mAb experiments were

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not exposed to a secondary antibody. Cells were then washed three times and incubated in the serum-free growth media without antibiotics for periods of 0, 18, or 24 hr prior to use for RT-PCR or ELISA.

Quantitative RT-PCR

Quantitative IL12 p40 RT-PCR was performed in cultured Jeg3 cells after cross-linking. Total RNA was isolated (RNeasy; Qiagen Inc., Valencia, CA, USA) and 2 μg of total RNA were subjected to reverse transcription (ReverTraAce; TOYOBO, Tsuruga, Japan) according to the manufacturer's instructions. A volume of 0.2–2 μL of each RT-reaction was used for quantitative PCR (Light Cycler system; Roche Diagnostics, Lewes, UK) using the following primer pairs: IL12 p40-forward 5′-GGTCACACTGGA CCAAAGGGACTATG-3′, -reverse 5′- ATTCTGCTGCC GTGCTTCCAAC-3′[24]; and β-actin-forward 5′- GA-AATCGTGCGTGACATTAAGG-3′, -reverse 5′- TCAG GCAGCTCGTAGCTTCT C-3′.[25] β-actin mRNA levels were quantified in each sample as an internal control for normalization. RT-quantitative PCR experiments were repeated at least three times.

ELISA for IL12 and IFNγ

Culture media was collected at 0, 18, and 24 hr after cross-linking (n = 4) and levels of secreted IL12 p70 or IFNγ were quantified using solid phase sandwich ELISAs (R&D Systems). A standard curve was produced using absorbance measurements at 450 nm for standard samples. Each unknown sample was similarly measured and plotted using this standard curve. IL12 and IFNγ secretion levels in the presence of the anti-β2GP1 antibody were normalized by those in the absence of the antibody and indicated as ratios.

Statistical Analysis

Quantitative PCR and ELISA data are presented as means ± standard deviations. All experiments were performed independently three times. mRNA or cytokine secretion levels were compared between cell lines and among time points using Mann–Whitney analyses. A P-value of <0.05 was considered significant.

Results

Cell-Surface Expression of PS and β_2GP1 Bound to CD1d

CD1d is an MHC-like glycoprotein that presents lipid antigens derived from pathogens and from self, the latter including phosphatidylserine (PS).[11] Phosphatidylserine can associate with β2GP1 at the surface of trophoblast cells.[26,27] The carrier involved in intracellular trafficking and transport of PS-β2GP1 complexes to the cell surface has not been identified. We hypothesized that the PS-β2GP1 complex is presented by CD1d in trophoblast cells. To test our hypothesis, we developed a trophoblast cell model that remained undifferentiated with prolonged *in vitro* culture, but stably expressed CD1d at the cell surface. Jeg3 cells are a commonly used model for extravillous trophoblast cells, but these cells do not endogenously express CD1d (Fig. 1a, green line). We transduced CD1d into Jeg3 cells using a retroviral expression system to establish the stable Jeg3/CD1d cell line with strong cell surface expression of CD1d (Fig. 1b, green line). Jeg3 and Jeg3/CD1d cells were triple-stained for PS, β2GP1, and CD1d and analyzed by flow cytometry (Fig. 1). Both PS (red lines) and β2GP1 (blue lines) were expressed on the cell surface of Jeg3/CD1d with signal peaks that overlapped that of CD1d (green lines). Neither PS nor β2GP1 was expressed on the cell surface of Jeg3 that lacked CD1d.

Using similar methodologies, we generated a cell line expressing a chimeric CD1 molecule comprised of the extracellular domains of CD1d and intracellular domain of CD1a (CD1d/a). As CD1a lacks a tyrosine in its cytoplasmic tail, downstream tyrosine-based signaling does not occur upon ligand binding to chimeric surface CD1d/a molecules.[13] The resultant cell line, called Jeg3/CD1d/a cells, can be used as a dominant-negative control in CD1d cross-linking experiments. As with Jeg3/CD1d cells, PS and β2GP1 were expressed on the surface of Jeg3/CD1d/a cells (Fig. 1c). Flow cytometry patterns for PS and β2GP1 are nearly identical in Jeg3/CD1d and Jeg3/CD1d/a cells, suggesting that PS and β2GP1 may form a complex at the cell surface and indicating that cell surface expression of PS and β2GP1 depends on the presence of the extracellular domains of CD1d.

To address biochemical interactions between CD1d and the PS-β2GP1 complex, an anti-CD1d mAb was

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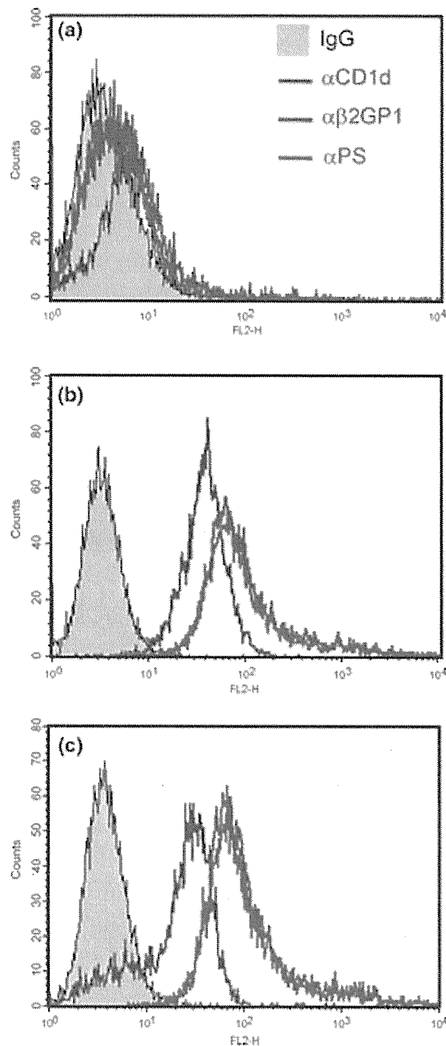

Fig. 1 Cell-surface expression of PS and β_2GP1 is CD1d-dependent. Jeg3 (a), Jeg3/CD1d (b) and Jeg3/CD1d/a (c) cells were triple stained with the anti-CD1d mAb (green), the anti-PS Ab (red) and anti-β2GP1 mAb (blue) and analyzed using flow cytometry. Background staining with isotype-matched control antibody is also shown (black). Histograms are representative of at least four separate experiments. (Blue lines (β2GP1) merged red lines (PS) in each panel of Fig. 1 because of the co-localization of the two molecules).

used to co-precipitate CD1d and its associated molecules from total cell lysates of Jeg3 and Jeg3/CD1d cells. These immunoprecipitants were then immunoblotted to detect β2GP1 (Fig. 2a). A 42-KDa band representing β2GP1 was present only in the Jeg3/CD1d cells, although immunoglobulin heavy chains were detected in all cells. Interaction between Cd1d and PS was similarly verified using co-immuno-

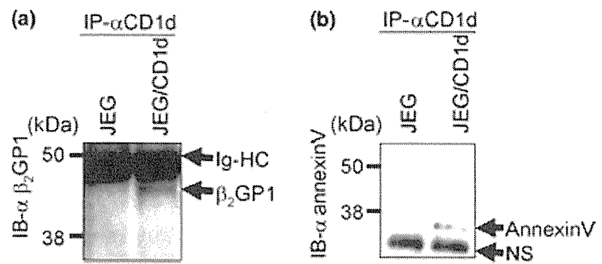

Fig. 2 The PS-β_2GP1 complex binds to CD1d. An anti-CD1d mAb was used to immunoprecipitate CD1d from Jeg3 or Jeg3/CD1d total cell lysates. (a) Immunoprecipitants were immunoblotted with an HRP-labeled anti-β_2GP1 mAb to detect β2GP1. An extra-band at 42 KDa, representing β_2GP1, was observed only in the Jeg3/CD1d lane although bands representing immunoglobulin heavy chains were noted in both lanes. (b) The immunoprecipitants were immunoblotted with an anti-Annexin V mAb to detect Annexin V, a PS-binding protein. An extra-band at 35 KDa, representing annexin V, was observed only in the Jeg3/CD1d lane although bands representing immunoglobulin light chains were noted in both lanes.

precipitation and Western blotting. Again, primary immunoprecipitations used an anti-CD1d mAb. As PS cannot be detected by Western blotting, these immunoprecipitants were immunoblotted with a mAb to Annexin V, a protein known to bind tightly to PS[23] (Fig. 2b). Although we could not detect PS directly, these biochemical data suggested the hypothesis that the PS-β2GP1 complex is expressed on the trophoblast cell surface and is bound to CD1d.

Ligation of Cell Surface CD1d Promotes IL12 Release from Trophoblast Cells

Ligation of surface-expressed CD1d promotes rapid but transient cytokine secretion from CD1d-bearing cells.[13,28,29] Such ligation can also occur upon interaction with iTCR-expressing iNKT cells and via antibody cross-linking of CD1d.[28] The cytoplasmic tail of CD1d, but not CD1a, bears a target domain for potential tyrosine kinase activity characterized by the tyrosine endocytic sorting motif (YXXZ).[13] CD1d ligation induces tyrosine phosphorylation in its cytoplasmic tail, subsequent intracellular signaling, and autocrine cytokine release from CD1d-bearing cells.[14,28] The 51.1 anti-CD1d mAb is often used for CD1d cross-linking and its use creates an *in vitro* model for CD1d ligation.[13,14] We have reported that cross-linking of CD1d using 51.1, when combined with secondary anti-Ig antibodies, promotes IL12 and IL15 secretion from reproductive tract epithelial

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cells.[29] Other groups have shown that similar CD1d cross-linking in monocytes and dendritic cells induces IL12 production [28] and IL12 is a known secretion product of normal human trophoblast cells.[30]

To address the function of CD1d in trophoblast cells, we examined CD1d ligation-induced autocrine cytokine production from Jeg3/CD1d cells. Jeg3 cells expressing the chimeric CD1d/a (Jeg3/CD1d/a cells) were used as dominant-negative control, as chimeric CD1d/a (extracellular CD1d and cytoplasmic CD1a) bears no target motif for phosphorylation. Both cell lines were first exposed to an anti-CD1d 51.1 mAb or to an isotype-control mAb. This was followed by exposure to a secondary anti-mouse IgG antibody cross-linker. The cells were then examined for IL-12 transcription (Fig. 3) using RT-PCR. Transcription of IL12 (p40) increased relatively rapidly (18 hr after cross-linking) in Jeg3/CD1d cells exposed to the CD1d-specific 51.1 mAb but did not increase in those exposed to isotype-control antibody. Although Jeg3/CD1d/a cells express cell-surface CD1d (shown in Fig. 1c), IL12 production was not induced in these cells upon exposure to anti-CD1d mAb or isotype control antibodies, indicating that IL12 production is mediated through pathways involving the cytoplasmic tail of CD1d.

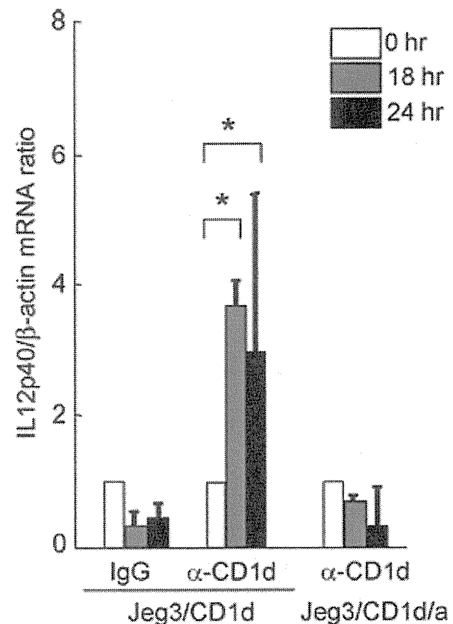

Fig. 3 CD1d cross-linking using anti-CD1d primary and anti-Ig secondary antibodies induces IL12 transcription from Jeg3/CD1d cells but not Jeg3/CD1d/a cells. Jeg3/CD1d cells or Jeg3/CD1d/a were exposed in culture to 10 μg/mL of the anti-CD1d mAb (51.1) or an isotype-control mAb for 1 hr. After washing, 10 μg/mL of goat anti-mouse Ig antibody was added as a cross-linker for 30 min to all cells. Cells were harvested at 0, 18, and 24 hrs after secondary antibody cross-linking. IL-12 (p40) mRNA levels were analyzed using quantitative RT-PCR and normalized to β-actin. Mean mRNA levels and standard deviations were plotted against time. Asterisks indicate those time point comparisons with statistical significance within a cell-line exposure ($P < 0.05$; $n = 4$).

An anti-β2GP1 Monoclonal Antibody Induces Autocrine IL12 Production from Trophoblast Cells by CD1d Ligation in the Absence of a Secondary Cross-Linker

Existing *in vitro* models for antibody cross-linking of CD1d requires use of primary and secondary antibodies.[13,28,29] Several investigators have demonstrated that one anti-β2GP1 antibody binds two cell-surface β2GP1 molecules.[31] In light of our finding that β2GP1 appears to be bound to cell-surface CD1d molecules via PS, we hypothesized that a single anti-β2GP1 antibody might ligate two CD1d molecules via their bound PS-β2GP1 complexes. This CD1d ligation would not require use of secondary antibodies and thereby could commonly occur *in vivo*. To address this hypothesis, Jeg3/CD1d and Jeg3/CD1d/a cells were exposed to anti-β2GP1 mAbs in the absence of secondary cross-linking antibodies. Post-exposure RT-PCR revealed that transcription of IL12 (p40) increased in Jeg3/CD1d cells 18 and 24 hr after exposure to anti-β2GP1 mAb alone (Fig. 4). Jeg3/CD1d cells exposed to isotype control and Jeg3/CD1d/a cells exposed to the anti-β2GP1 mAb exhibited no change in IL12 transcription when analyzed up to 24 hr post-exposure. Notably, the increase in IL12 transcription after anti-β2GP1 mAb exposure was greater than that seen after combined anti-CD1d and secondary antibody exposure (Fig. 3).

Autocrine Secretion of IL12 from CD1d-Bearing Trophoblast Cells upon CD1d Ligation

To examine autocrine IL12 production from CD1d-bearing JEG3 cells after CD1d ligation, protein secretion into the culture media was assessed by ELISA (Fig. 5). Jeg3, Jeg3/CD1d, or Jeg3/CD1d/a cells were exposed to an anti-CD1d mAb (51.1) then to secondary antibody cross-linker as described previously. In separated cultures, these same cells were also

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