2 クローン黎明期

Driesch らの卵割球を分離し発生個体を観察する実験より，少なくともごく初期の各卵割球は遺伝的等価であるという知見が得られていたが，さらに発生が進行した個体の細胞ではどうか．その問いに答えたのが Jacques Loeb（1894）と Hans Spemann（1914）である．Loeb は，ウニ受精卵を低浸透圧生理食塩水に置くことで無核細胞質と有核細胞質部分に分け，発生が進行した有核部分の1細胞核を無核細胞質へ移す実験を行った．Spemann は，娘の毛髪を使いイモリ受精卵を Loeb 同様に無核，有核部分に分け，16細胞期に至った有核部分の1細胞核を無核部分へ移行させ発生させたところ，両胚とも正常な個体に発生した．これにより，少なくとも初期発生において遺伝形質は脱落するのではなく，各細胞のゲノムは均一で全能性があることが判明した．Spemann はその後，カエル胚を使った移植実験による発生の運命付けに関する数々の発見をし，胚発生におけるオーガナイザーに関する一連の業績により1935年ノーベル賞を受賞した．

それでは，細胞核を卵に移植して発生をさせるという現在のクローニング技術の原型はいつ頃から行われていたのであろうか．Di Berardino 女史によると Rauber（1886）が最初であるらしい[3]．Rauber は，細胞核そのものが遺伝形質機能を持っているか証明するためにカエル受精卵を使って細胞核を置換する実験を行った．結果は，残念ながら核置換卵は発生しなかった．しかし Spemann は，完全に分化した細胞を除核した卵子へ入れると正常発生が可能かもしれないことを記述している[3]．20世紀に入り，実験発生学においても胚を扱うことのできる器具の発達や，技術を持った研究者が台頭してきた．1940年代に米国ペンシルバニア州フォックスチェイス癌センターの Robert Briggs は，体細胞核の遺伝的等価性（受精卵と体細胞では核ゲノムは同一であること）を解析するためカエルを使った研究を行っていたが，なかなか思うような成果が得られずにいた．1950年にニューヨーク大学の大学院生 Thomas J. King が Briggs の研究室に入って歴史は動いた．King は当時，ミクロの胚操作技術ではパイオニアの一人である Robert Chambers より胚操作術の手ほどきを受け，技術的に十分なものを持っていた．そこで，ドナー細胞にダメージを加えない移植法などサイトジェネティック基礎研究をこなしつつ，細胞核移植法により1952年までに現在の体細胞核移植法の原型となる方法を確立した．カエル胚の細胞よりクローン個体（オタマジャクシ）作成に成功した．初めてクローン胚が胞胚期まで発生したのを見た Briggs は研究室に響き渡る歓声の声を上げ，研究所中の人々が顕微鏡をのぞきに来た．そして，大騒ぎが一段落ついた時，King が顕微鏡をのぞいてみると，せっかくできた初のクローン胞胚はつぶれていたそうで，周りの人は「もしこのクローンが本当なら，君ならまた作れるよ」とひどく落ち込んでいた King を慰めたそうである．1952年には，Briggs と King は核移植法を細胞核分化能解析に用いる以外にも，癌，細胞老化，免疫学，そして遺伝子発現の分子レベルでの研究など広く応用できることを示唆している．

その後，John Gurdon らはオタマジャクシの腸や成体の皮膚の細胞を使ったクローンを成功させてきた．1970年代までに世界中の研究者が両生類クローンの成功を報告してきたが，幼生，成体からの細胞核移植により得られたクローン個体は成体まで発生することはなかった．その後，細胞核全能性に関する研究は昆虫，魚類，そして哺乳動物を使用したクローニング研究へと広がりを見せていった．

3 哺乳動物のクローン

Briggs と King がカエルでクローンの成功を伝えてから約30年後の1980年代初頭，哺乳動物クローンの成功が報告されてきた．これほどの時間がかかった理由は，一つに哺乳動物卵は両生類に比べて非常に小さく胚操作に対して脆弱であり，その卵に合わせて顕微胚操作術，顕微鏡システ

ム，除核術，卵活性化法，核移植術等を改良しなくてはならなかったからである．さらに，卵の体外培養システムと培養液の改善を待たなければならなかったこと等が挙げられる．哺乳動物でのクローン研究において当初の目的は2つあった．1つには，他の種同様に初期発生の段階での細胞核の全能性に関して答を出すことであり，2つ目は畜産動物の改良であった．実験動物マウスを用い，不活性化したセンダイウイルスを細胞融合に応用した細胞核移植実験は1969年頃から行われていたが，ほとんど異常な卵割期発生で止まっていた．James McGrath と Davor Solter はマウスを用いて初めて核移植術を成功させた[4]．受精卵より雌雄両前核を抜き取り，除核した卵と不活性化したセンダイウイルスにより融合させた．その結果，16％の率で正常な産仔への発生が認められた．彼らはまた，この方法を応用して雌性胚と雄性胚発生を解析することで，哺乳類の発生には雌雄両ゲノムが必要であることを証明し，インプリンティングメカニズムの研究に多大なる貢献をした[5]．マウス初期胚および胎児由来の細胞を使った体細胞核移植研究で貴重な成功例を報告してきたのは，日本人の角田と河野であった．一連のマウスクローン実験より，受精卵から8細胞期胚までの細胞核は全能性があり，2細胞期と4細胞期の単離した割球は成体まで発生することができる．そして，ドナー細胞のゲノムのリプログラミングが移植後の卵内で起こることが報告された．また，マウスの他にウシ，ヒツジ，ヤギ，ブタ，ウサギでクローン研究が広く行われていった．

遠年ときた生物学上の大きな問いに Briggs と King をはじめとした研究が答を出したのに，その後もクローン研究がどんどん広がりを見せてきたのは何故なのだろうか．言い換えると，クローン研究をやる意味はどこにあるのだろうか．これまでの研究成果で受精卵からの発生では遺伝形質（ゲノム）が失われ，分化した細胞になるのではないことと，細胞核（成体では答が出ていなかったが）は受精卵と等しいゲノム情報を保持していることがわかった．この時点で，さらにクローン研究をやる理由は少なくとも3つ考えられる．1つは，成体では各細胞は高度に分化した細胞，つまりその組織特異的な遺伝子発現があり，特化した働きを持つ分化細胞ではゲノムの脱落，変異が本当にないのであろうか．2つ目は，分化細胞がもしゲノムの脱落，変異がない，つまりゲノムは受精卵と等価であるとするなら，分化細胞核も受精卵同様ゲノム全能性（genomic totipotency）を有する．通常，心筋細胞のような細胞が受精卵に戻ることはない．しかし，これまでのクローン研究が示したように，分化細胞核でさえ卵の中に入れると発生時計をもう一度リセットし，全能性を引き出すことができる可能性が出てきた．卵が分化細胞ゲノムをリセットできる機能，つまり細胞核リプログラミング（genomic and epigenetic reprogramming）とはどのような仕組みで起きているのか，その正体は何か，それを解明し人為的に応用できれば，組織を再生することが可能になるはずである．3つ目は，当初のクローン研究の目的の一つに畜産動物の生殖応用が挙げられていた．クローンの効率を上げることは，クローン研究が現場へ応用されるために非常に重要であったと考えられる．現に，1980年代から1990年代にかけて細胞核リプログラミングの研究は盛んに行われるようになってきた．リプログラミングが解明されれば体細胞クローンも可能になるであろうと考えられたのも一因である．しかし，リプログラミングメカニズムに関して分子レベルではほとんど何も解明されていなかった．

4 体細胞クローン動物の誕生

1997年，遂に体細胞クローンの成功が報告された．Ian Wilmut らは，成体雌羊の乳腺細胞を除核した未受精卵と融合させ活性化させた後仮親へ移植したところ，クローン羊の「ドリー」が誕生した．翌年より他の動物でも体細胞クローン動物の成功例が相次いで報告された．1998年には柳町と若山が実験動物であるマウスで，角田

らはウシで体細胞クローン動物の作出を報告した．現在までにヤギ，ブタ，ネコ，ウマ等で成功している．クローン動物作出の成功は，クローン技術が様々な分野で応用できる可能性を示した．Wilmut らはドリーを作成した技術と遺伝子組換え技術を使い，血友病の治療に必要なヒト血液凝固因子（第IX因子）遺伝子を組み込まれたクローン羊「ポリー」を作成した．羊乳から第IX因子タンパク質を精製し，治療薬として利用することを目指した．旧科学技術庁はクローンの応用例として，実験用動物の革新，希少動物の保護・再生，食料（畜産）の安定供給，医薬品の製造，移植用臓器の作成等を挙げている．しかし，体細胞クローン動物が作成されてわかったことは，クローン胚の個体発生率が非常に低いこと，流産や胎児奇形率が高いこと，胎盤に組織的・機能的な異常が認められること，クローン動物の中には短命な個体が出てきたこと等の問題が出てきたことである．クローン研究における社会の注目は，当初の目的である発生過程におけるゲノム不変やリプログラミング解析よりも，ヒト生殖応用への危惧へと集まった．クローン動物作成の土台となるマイクロインジェクションシステムは，生殖補助医療での顕微授精システムとほぼ同様であることで，世界中でクローン技術のヒトへの安易な応用が危惧されたため，ドリーの報告以来すぐさま世界中でヒトクローン禁止の法律が整備されていった．研究者達もヒト生殖応用禁止を訴えている[6]．ヒトクローン禁止の理由として，クローン胚，胎児の異常率が高いこと，多数の正常卵の必要性が挙げられているが，母体の保護も付け加えるべきである．流産率が高いだけではなく，これまでのクローン動物作成において胎盤の形態的および組織学的な異常はほぼ 100 %認められている．私達が行ったクローン胚の胎盤での胎盤特異的遺伝子発現を解析した結果，すべてのクローン胎盤で遺伝子発現の異常が認められた．ヒトでは胎盤の機能低下・不全による重篤な妊娠合併症が知られているので，ヒトクローン胚の子宮への移植は非人道的行為であると認識するべきである．

5 体細胞核移植術の広がる可能性

1952 年に Briggs と King が示唆した通り，クローニング技術はクローン個体作成目的以外の様々な分野へ応用されていった．例えば，石野と小倉らはこれまでの技術では不可能であった始原生殖細胞発生段階に起きるゲノムインプリンティング確立の解明にクローニング技術を応用することで重要な発見をしてきている[7]．基礎研究においてクローニング技術が非常に有用なツールとなることが理解されていたが，“クローン”とつくことで社会から本来の研究に対してネガティブなイメージがつくのを避けるために，前述した Vogelstein らが “Please Don't Call It Cloning !” と訴えたわけである．これは何も基礎研究に対してだけでなく，次に述べる再生医療応用への影響もある．

クローン羊ドリーが報告された翌年の 1998 年，ヒト ES 細胞樹立の成功が発表された[8]．これで一気に体細胞核移植法とヒト ES 細胞の再生医療への応用が現実味を帯びてきたのである．当初この方法は，Therapeutic Cloning と呼ばれていたが，現在では Cloning という単語を使わない Cell Replacement Therapy（CRT）と呼ばれることが多い．ES 細胞は胚盤胞の内部細胞塊より樹立される細胞で，身体のすべての細胞に分化することができる能力（分化多能性）を保持したまま体外培養系で無限に増殖することができる稀有な細胞である．難治疾患で機能不全あるいは低下した組織を，ES 細胞を用いた細胞移植により機能を改善させようとする細胞移植療法において，他人の細胞を移植した場合拒絶されるか，副作用の強い免疫抑制剤の服用は避けられない．そこで，体細胞核移殖法を応用し，患者自らの核ゲノムを持つ ES 細胞を樹立し細胞移植へ用いるのである（CRT）．この CRT に関する研究では，韓国の黄禹錫教授の研究ねつ造と生命倫理学的手続きの不正によりその必要性自体を問われているが，米国や英国の研究グループは非常に厳しい倫理手続

きのもと，ヒト体細胞核移植法による胚の作成とES細胞樹立研究の準備を進めている．体細胞核移植法によるヒトES細胞の作成は，CRTのみに向かっているのではなく，病気モデルのES細胞を作成することも大きな意義を持っている．現在多くの慢性変性疾患では有効な治療法のない，例えば，パーキンソン病患者の細胞を用いて体細胞核移植による胚からES細胞を樹立する．ゲノム上にその病気の何らかの変異を持ったと考えられるES細胞から神経へ分化誘導させ，正常なES細胞からの神経誘導と分子あるいは機能レベルで詳細に比較検討する．このヒト細胞による体外実験系によりパーキンソン病の病因解明にあたるとともに，治療薬の開発や新しい治療法の開発を行うことができる．

生殖補助医療において体細胞核移植術の応用の可能性はあるのだろうか．生殖細胞の代わりに体細胞ゲノムを半数化させて生殖補助医療へ応用する可能性も指摘されていたが，私たちはそのサイトジェネティックな明らかな不完全性を報告している[9]．他には，体細胞核移植術ではないが，同様なマイクロマニピュレーション技術を使用した，GV卵の核置換や着床前期診断で行う割球あるいは胚盤胞期の細胞採取に応用できる．我が国においてクローニング技術をヒト生殖に応用することを禁止する法律「ヒトに関するクローン技術等の規制に関する法律」が平成13年より施行されている．ヒト体細胞核移植法のES細胞樹立研究に関しては，国はその必要性のあることを認識し，総合科学技術会議において，平成16年7月に「ヒト胚の取扱いに関する基本的な考え方」（意見）が取りまとめられ，人クローン（体細胞核移植）胚の研究目的の作成・利用については，他に治療法の存在しない難病等のための再生医療の研究目的に限って認め，クローン技術規制法に基づく特定胚指針の改正等により必要な枠組みを整備すべきとされた．これを受けて，人クローン胚研究利用作業部会委員会は毎回委員会を公開で行い，さらに平成18年には関係団体や一般の方から意見を聴取する会を2回行い，「人クローン胚の研究目的の作成・利用のあり方について―人クローン胚研究利用作業部会中間取りまとめ―」を発表し，特定胚指針等の改正に向けて基本的な考え方について報告している．胚盤胞から樹立したES細胞が再生医療応用で社会の関心を集め，卵子・卵・初期胚の存在は，生殖医療のみならず再生医療への関わりを通し，今までよりも広く社会の関心を集めてきている．

体細胞核移植法は基礎研究での応用から医学研究，そして再生医療への応用等，新たな可能性を広げている．しかし，医療応用の可能性が広がるとともに社会との関わりが密接となる．医療と社会との関わり方で重要になるのは，生命倫理観の認識とそれに基づいた行動である．体細胞核移植研究が適切に評価され，社会に貢献することを期待している．

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Selective increase in high molecular weight adiponectin concentration in serum of women with preeclampsia

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Abstract

Total adiponectin concentrations have been shown to increase in serum of preeclamptic women. However, variance of concentrations of different isoforms has not been studied, despite the emerging notion that high, medium and low molecular weight adiponectin exert different functions. We have determined serum concentrations of each adiponectin isoform using a newly developed enzyme immunosorbent assay. High molecular weight (HMW) adiponectin concentrations were significantly higher in women with preeclampsia ($n = 14$; median, 11.2 μg/ml; interquartile range, 9.2–15.8) compared to normal pregnant women ($n = 14$; 6.8 μg/ml, 5.4–10.7; $P = 0.04$). In contrast, medium molecular weight and low molecular weight adiponectin concentrations were substantially equal between the groups. The ratio of HMW adiponectin to total adiponectin was also markedly higher in preeclamptic women (52.3%, 49.5–58.7) than control women (43.0%; 39.8–48.0; $P = 0.004$). Taken together with other reports our findings imply a physiological feedback response to minimize endothelial damage in preeclamptic women.
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Keywords: Adiponectin; Preeclampsia; Molecular weight; serum

1. Introduction

Preeclampsia is one of the common causes of maternal death and a major contributor to both maternal and fetal morbidity. Its clinical manifestations, i.e. hypertension, proteinuria and edema, are caused by widespread endothelial damage and dysfunction throughout maternal circulation (Redman and Sargent, 2005; Roberts et al., 1989). Several candidate molecules that induce the endothelial dysfunction, sVFGFR-1/sFlt-1 being the most probable (Koga et al., 2003; Levine et al., 2004), have been suggested; however, the etiology remains elusive.

Adiponectin is a pleiotropic cytokine with anti-diabetic, anti-inflammatory and anti-atherogenetic properties (Brakenhielm et al., 2004; Goldstein and Scalia, 2004; Kadowaki and Yamauchi, 2005; Takemura et al., 2005a,b; Yokota et al., 2000). Experimental data have demonstrated that adiponectin acts as an endogenous modulator, exerting a number of vascular-protecting activities (Arita et al., 2002; Matsuda et al., 2002; Okamoto et al., 2002; Ouchi et al., 2000, 2001). In addition, the adiponectin concentration was reported to be significantly lower in hypertensive men than in normotensive men regardless of insulin resistance (Iwashima et al., 2004).

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Therefore, circulating adiponectin concentrations were hypothesized to decrease in women with preeclampsia. Insulin-resistance associated with preeclampsia (Kaaja et al., 1999; Solomon and Seely, 2001) also sustains the assumption, because serum adiponectin concentrations are inversely related with insulin resistance (Matsubara et al., 2002). Contrary to expectation, however, recent studies have shown that adiponectin concentrations were increased by 47% (Ramsay et al., 2003), 87% (Naruse et al., 2005) and 30% (Kajantie et al., 2005) in preeclamptic compared to normotensive women with similar body mass index. The reason of this paradoxical finding has been suggested to be due to compensatory mechanisms in women with preeclampsia.

Recent studies demonstrated that adiponectin circulates as a trimer (low molecular weight, LMW), hexamer (medium molecular weight: MMW) or high molecular weight (HMW) form (Waki et al., 2003). Each isoform has been shown to play different biological roles (Bub et al., 2006; Kobayashi et al., 2004; Matsuda et al., 2002; Wang et al., 2005). Remarkably, only the HMW form protects endothelial cells from apoptosis (Kobayashi et al., 2004). In addition, the proportion of different isoforms is altered in various pathological and physiological conditions (Bobbert et al., 2005; Kobayashi et al., 2004; Lara-Castro et al., 2006; Pajvani et al., 2003). Therefore, isoform abundance should be evaluated when examining the role of adiponectin in different disease states.

To date, all isoforms were recognized by commercial adiponectin assay kits, including those used in studies showing the paradoxical increase of adiponectin concentration in women with preeclampsia (Kajantie et al., 2005; Naruse et al., 2005; Ramsay et al., 2003). Generally, these assays were used to measure the total adiponectin concentrations. In contrast, a newly developed adiponectin assay system can measure separately the concentration of each isoform. To address further the relevance of adiponectin in preeclampsia, we have measured serum concentrations of different adiponectin isoforms in women with preeclampsia using the new assay system.

2. Materials and methods

2.1. Subjects

A total of 28 pregnant women with ages of 28–38 years, with ($n = 14$) and without ($n = 14$) preeclampsia, were included in this study. Their gestational ages ranged from 26 to 40 weeks. Preeclampsia was defined as blood pressure >140/90 mmHg with proteinuria of either >100 mg/dl by urine analysis or >300 mg in a 24-h urine collection. None of the patients with preeclampsia had any prior history of hypertension or renal disease. Control subjects with body mass index (BMI) similar to the group with preeclampsia were collected from women who had no hypertension, proteinuria nor edema. None of the subjects had gestational diabetes or a history of metabolic disease. Each group included two women with gestational ages earlier than 32 weeks at the time of sampling. All women carried a single fetus.

The experimental procedures were approved by the Institutional Review Board, and signed informed consent was obtained from each woman.

2.2. Measurements

Blood samples were collected from women with preeclampsia soon after the manifestation of the disease and before commencing any medication. Blood samples were obtained after an overnight fast. Serum was separated by centrifugation and stored at −80 °C before use. Concentrations of total, HMW, MMW and LMW adiponectin in serum were measured using a commercially available enzyme-linked immunosorbent assay kit (Daiichi Pure Chemicals, Tokyo, Japan), according to the manufacturer's protocol. The minimum detectable level was 75 pg/ml. The intra- and inter assay coefficient of variations were both less than 10%.

2.3. Statistical analyses

Maternal ages, gestational ages at the time of sampling, parity, BMI before pregnancy and BMI at test between preeclamptic and control women were compared using Student's t-test. Serum adiponectin levels were compared using Mann–Whitney U-test. $P < 0.05$ was considered to be statistically significant.

3. Results

As shown in Table 1, maternal age, gestational age, parity, BMI before pregnancy and BMI at test were substantially equal between the control and preeclamptic women.

As depicted in Fig. 1, serum concentrations of total adiponectin appeared to be higher in preeclamptic than control women, although this difference was not statistically significant ($P = 0.13$). Notably, concentrations of HMW adiponectin were significantly higher in preeclamaptic women than in the control women ($P = 0.04$). Concentrations of both MMW and LMW adiponectin were comparable between preeclamptic and control women ($P = 0.26$ and 0.65, respectively).

Table 1
Characteristics of study subjects

	Control ($n = 14$)	Preeclampsia ($n = 14$)	Statistical significance
Maternal age (years)	32.7 ± 3.0	33.0 ± 2.9	NS
Gestational age (weeks)	35.0 ± 4.0	35.2 ± 3.7	NS
Parity (number)	0.21 ± 0.43	0.21 ± 0.43	NS
BMI before pregnancy (kg/m^2)	20.9 ± 1.6	20.6 ± 3.8	NS
BMI at test (kg/m^2)	24.1 ± 2.0	24.3 ± 4.3	NS
Systolic blood pressure (mmHg)	92.4 ± 10.8	158.5 ± 26.1	$P < 0.0001$
Diastolic blood pressure (mmHg)	52.4 ± 10.0	93.5 ± 14.0	$P < 0.0001$

Values are mean ± S.D. NS: not significant, $P > 0.05$.

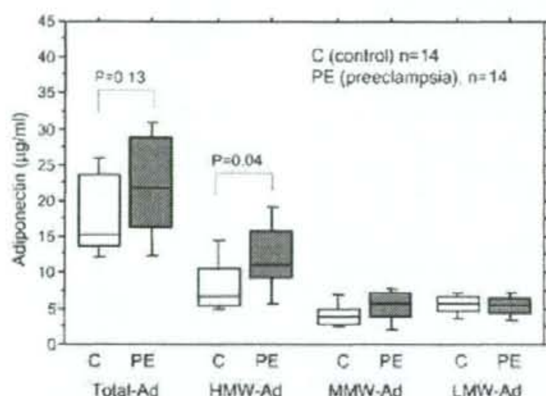

Fig. 1. Serum concentrations of total, HMW, MMW and LMW adiponectin in control ($n = 14$) and preeclamptic ($n = 14$) women. Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent medians. The whiskers represent the 10th percentile at the lower limit and the 90th percentile at the upper limit.

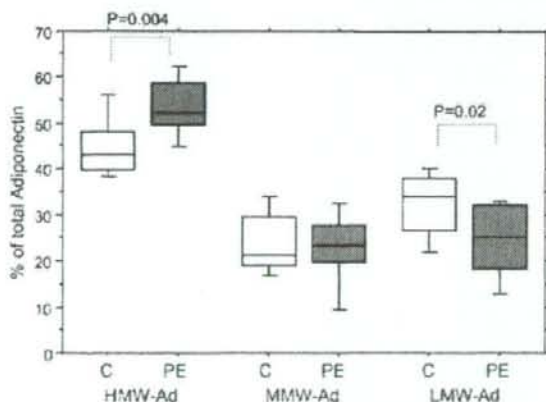

Fig. 2. The concentrations ratios of HMW, MMW and LMW adiponectin to total adiponectin in control ($n = 14$) and preeclamptic ($n = 14$) women. Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent medians. The whiskers represent the 10th percentile at the lower limit and the 90th percentile at the upper limit.

Fig. 2 illustrates the concentration ratio of each isoform to total adiponectin. The ratio of HMW adiponectin is markedly higher in preeclamptic than control women ($P = 0.004$). The ratio of MMW adiponectin is comparable between the groups ($P = 0.75$), and the ratio of LMW adiponectin is lower in preeclamptic than in the control women ($P = 0.02$).

4. Discussion

A significant increase of HMW adiponectin concentration was observed in the serum of women with preeclampsia in the present study. Similarly, the ratio of HMW to total adiponectin was increased. Given that the only HMW form has vascular-protective features (Kobayashi et al., 2004), our findings, like previous findings that used a total adiponectin assay, seem paradoxical to the assumption that adiponectin causes preeclampsia.

The increase of serum adiponectin concentrations in women with preeclampsia is speculated to be a defensive reaction of the body. Vascular-protective effects of adiponectin may alleviate endothelial damage and dysfunction induced by preeclampsia. Women with preeclampsia are characterized by insulin-resistance (Kaaja et al., 1999; Solomon and Seely, 2001; Wolf et al., 2002) and chronic systemic inflammatory responses (Redman et al., 1999). As adiponectin has also anti-diabetic and anti-inflammatory features, the increase in serum adiponectin concentrations in preeclamptic women seems feasible to compensate for these detrimental conditions associated with preeclampsia.

The mechanism of the increase in serum adiponectin in preeclamptic women is unknown. The main source of circulating adiponectin has been claimed to be fat tissue; however, a recent study has shown that mean adiponectin mRNA levels are similar in abdominal subcutaneous adipose tissue in preeclamptic and control women (Haugen et al., 2006). Whether the placenta produces adiponectin or not is controversial at this time (Caminos et al., 2005; Corbetta et al., 2005). It is unlikely that the increase of HMW adiponectin in preeclamptic women was due to renal failure since serum creatinine levels were within the normal range (0.67 ± 0.16 g/dl, mean $\pm$ S.D.).

In contrast to elevated adiponectin concentrations in women with preeclampsia, first trimester hypoadiponectinemia is reported to increase subsequent risk of hypertensive disorders during pregnancy (D'Anna et al., 2005). Although the finding appears contradictory, it is understandable that women with hypoadiponectinemia in early pregnancy are more vulnerable to vascular and metabolic stress of ongoing pregnancy and are predisposed to develop preeclampsia. Nevertheless, as shown in another study, adiponectin levels were comparable after delivery in preeclamptic compared to normotensive pregnancy (Kajantie et al., 2005). This suggests that elevated adiponectin is related to the pathophysiology of preeclampsia rather than an inherent property in women with a history of preeclampsia.

Multiple lines of evidence indicate that levels of different molecular weight forms of adiponectin in circulation are distinctively regulated. Body weight inversely correlated with HMW and positively with LMW (Bobbert et al., 2005), while weight reduction increases HMW but not LMW (Bobbert et al., 2005; Kobayashi et al., 2004). HMW concentrations are correlated also with insulin sensitivity (Lara-Castro et al., 2006), and are significantly reduced in response to a systemic increase of insulin (Pajvani et al., 2003). Interestingly, female mice display significantly higher concentrations of HMW in serum than males (Pajvani et al., 2003), and testosterone selectively reduces HMW by inhibiting its secretion from adipocytes (Xu et al., 2005). However, in view of increased androgen levels in preeclampsia (Troisi et al., 2003), androgen does not seem to be a main regulator to increase serum HMW adiponectin concentrations in preeclampsia.

To date, most studies that have examined various activities of adiponectin with regard to different molecular weight forms suggest that mainly the HMW form has significant biological roles. The anti-apoptotic effect toward human umbilical vein endothelial cells was observed only with the HMW form (Kobayashi et al., 2004). The HMW form, but not the LMW form, is inhibitory for prostate cancer cell growth (Bub et al., 2006). The HMW and MMW forms, but not the LMW, bind to T-cadherin expressed in endothelial and smooth muscle cells (Hug et al., 2004). Together with these findings, our observation implies that the HMW form plays some specific role in women with preeclampsia.

Consistent with the notion that the main activities of adiponectin are attributable to the HMW form, several studies advocate the importance of the HMW form as a percentage of total adiponectin (percentage of HMW adiponectin [S_A] index) in relation to pathophysiology of diseases. The S_A index was a better determinant of glucose intolerance than measurements of total adiponectin (Fisher et al., 2005; Pajvani et al., 2004). The S_A index was significantly lower in patients with coronary artery disease than control subjects (Kobayashi et al., 2004). Similarly, the S_A index was remarkably higher in women with preeclampsia than the control in our study, supporting the significance of the index.

The present study has limitations due to the small sample size. No significant correlation of serum HMW

adiponectin concentrations was detected with either BMI or blood pressure in the present study, perhaps due to the sample size. In addition, changes of serum HMW adiponectin concentration over time remain to be elucidated, considering that serum total adiponectin concentrations decrease after delivery (Kajantie et al., 2005). Therefore, a further study with a larger sample size is required to investigate more precisely the implication of adiponectin in preeclampsia.

In summary, this study has demonstrated that serum concentrations of the HMW form of adiponectin are significantly increased in women with preeclampsia, whereas the MMW and LMW forms are comparable. Given the vascular-protective activities of the HMW form, the increase observed in preeclamptic women might be a physiological feedback response to minimize endothelial damage.

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The Expression and Possible Roles of Chemokine CXCL11 and Its Receptor CXCR3 in the Human Endometrium

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IFN-γ secreted by a human embryo and trophoblast cells during implantation is suggested to play an important role in implantation and pregnancy. In the present study, we explored expression and possible functions of CXCL11, a CXC chemokine strongly induced by IFN-γ, and its receptor CXCR3 in the human endometrium. Secreted CXCL11 protein was not detected in cultured endometrial stromal cells (ESC) but was detected in cultured endometrial epithelial cells (EEC). IFN-γ stimulated the protein levels of CXCL11 in a dose-dependent manner in EEC and ESC. CXCL11 secreted from EEC with 100 ng/ml IFN-γ was 220-fold of the control, and 100-fold as compared with that secreted from ESC with the same dose of IFN-γ. CXCR3 was expressed in EEC, ESC, and trophoblast cells. Addition of IFN-γ to EEC increased the chemotactic activity of its culture medium to trophoblast cells and T cells, and the effect was suppressed by immunoneutralization with Abs of three CXCR3 ligands, including anti-CXCL11 Ab. CXCL11 significantly increased BrdU incorporation of ESC, which was inhibited by a p42/44 MAPK pathway inhibitor PD98059. In contrast, CXCL11 significantly decreased BrdU incorporation and increased the release of lactate dehydrogenase and the positive staining of annexin V in EEC. These findings suggest that IFN-γ promotes implantation by stimulating EEC to produce CXCL11, which induces migration of trophoblast cells and T cells, proliferation of ESC, and apoptosis of EEC. ***The Journal of Immunology*, 2006, 177: 8813–8821.**

Implantation occurs on days 20–24 of a regular 28-day menstrual cycle which is compared to ~6 or 7 days after fertilization. Human implantation includes three stages which are called apposition, adhesion, and invasion. The initial unstable contact of the blastocyst to the uterine wall is apposition which occurs most commonly in the fundal wall of the uterus. The next stage, adhesion, is characterized by increased physical interaction between the blastocyst and the uterine epithelium. Subsequently, the third stage of implantation, invasion, begins and trophoblast cells infiltrate the uterine epithelium (1).

The endometrial epithelium is an important element where the molecular interactions between the embryo and the endometrium are initiated (2–4). A specific molecular cross-talk between embryo and endometrium has been reported during the human implantation process (5). Cytokines, such as IL-1, LIF, CSF-1, and IL-8, and their specific receptors, which are expressed in the endometrium and the embryo, are suggested to be involved therein (5–7).

IFN-γ secreted by the preimplantation embryo and endometrial leukocytes is suggested to play an important role in the process of implantation in humans (8–10). IFN-γ produced from the human embryo is highest when it develops to blastocyst and reaches a point of apposition in the uterus (8). This finding implies that IFN-γ may have some roles in the initial stages of implantation.

IFN-γ is known to strongly induce three CXC chemokines, CXCL9 (monokine induced by IFN-γ), CXCL10 (IFN-γ-inducible protein of 10 kDa), and CXCL11 (IFN-γ-inducible T cell α chemoattractant), in a range of cell types (11–14). These chemokines exert their effects through a shared receptor called CXCR3 (12, 13).

Recent studies demonstrated that these three chemokines were expressed in both pregnant and nonpregnant endometrium (15–18). In cultured endometrial stromal cells, IFN-γ, IL-1β, TNF-α, and LPS stimulated CXCL10 production (16) and progesterone induced secretions of CXCL9 and CXCL10 (17). Notably, a recent study showed that CXCL10 stimulates the migration and attachment of ovine trophoblast cells (19). In contrast, CXCL11 in the endometrium has been poorly studied despite its highest binding affinity to CXCR3 among the three chemokines (12).

Based on these findings, we speculated that IFN-γ could regulate implantation through CXCL11 production in the endometrium. In the present study, we first showed IFN-γ-induced production of CXCL11 in endometrial cells. We then examined the expression of CXCR3 in endometrial cells and trophoblast cells and effects of CXCL11 on these cells, aiming to assess the possible roles of CXCL11 and CXCR3 in implantation.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics, magnesium sulfate ($MgSO_4$), and streptokinase were purchased from Sigma-Aldrich. DMEM/Ham's F12 medium (DMEM/F12), RPMI 1640 medium, medium 199, 0.25% trypsin, and 0.25% trypsin/EDTA were obtained from Invitrogen Life Technologies. Charcoal-stripped FBS was obtained from HyClone. A specific inhibitor of ERK (MEK)-1, PD98059, was obtained from Calbiochem. Rabbit polyclonal Abs to human CXCL9 were obtained from PeproTech. Rabbit polyclonal Abs to human CXCL10 and human CXCL11 were obtained from BioVision. Rabbit polyclonal Abs to human total p42/44 MAPK and phospho-p42/44 MAPK were obtained from New England Biolabs. Anti-rabbit HRP secondary Ab and Ficoll-Paque Plus (1.077 g/ml) were obtained from Amersham Biosciences. Mouse monoclonal anti-human CXCR3 Ab, human

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recombinant IFN-γ, human recombinant CXCL11, and human recombinant IL-2 were obtained from R&D Systems. Isotype control mouse IgG1, isotype control rabbit IgG, and mouse mAbs to human vimentin, human cytokeratin, and human CD45 were obtained from DakoCytomation. Mouse mAbs to human cytokeratin type 7 were obtained from Immunologicals Direct. FITC-conjugated anti-mouse IgG (H+L) Ab was obtained from Beckman Coulter. DNase I was obtained from Takara.

Sources of tissues

Endometrial tissues were obtained from a total of 60 patients (aged 40.3 ± 4.9 years, mean ± SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroid without endometrial pathologies. Although the relatively high ages of the subjects in the reproductive age range and the pathologies of the myometrium may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue of healthy young women. All of them had regular menstrual cycles and had not received hormone therapy for at least 6 mo before surgery. The specimens were dated according to the patients' menstrual history and standard histological criteria by Noyes et al. (20). Placental tissues between 5 and 7 wk of gestation were obtained at elective termination of pregnancy. PBMC were obtained from normal volunteer donors. The Institutional Review Board of the University of Tokyo approved this study and written informed consent for use of the tissue was obtained from each woman. The tissues collected under sterile conditions were processed for primary cell cultures.

Isolation, purification, and culture of endometrial epithelial cells (EEC),[2] endometrial stromal cells (ESC), T cells, and trophoblast cells

Isolation and culture of human EEC and ESC was as described previously (21–24). Endometrial tissues were minced and incubated in DMEM/F12 containing 0.25% type I collagenase and 15 U/ml DNase I for 60 min at 37°C. The resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (BD Biosciences). Endometrial epithelial glands which remained intact were retained by the strainer, whereas dispersed ESC passed through the strainer into the filtrate. ESC in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 10% FBS and antibiotics. ESC were plated in a 100-mm culture plate and kept at 37°C in a humidified 5% CO_2/95% air atmosphere. At the first passage, the cells were plated at a density of 2×10^5 cells/well into 12-well culture plates for the experiments of RT-PCR, Western blotting, and ELISA, or at the density of 1×10^4 cells/well into 96-well culture plates for the experiments of cell proliferation assay. Cells enriched with endometrial epithelial glands were collected by backwashing the strainer with DMEM/F12, plated in a 100-mm plate, and incubated at 37°C for 60 min to allow contaminated stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment with culture plates. EEC at a density of $\sim 2 \times 10^5$ cells/well in 12-well culture plates were used for the experiments of RT-PCR, Western blotting, ELISA, and assays of cytotoxicity and apoptosis, and EEC at the density of $\sim 1 \times 10^4$ cells/well in 96-well culture plates were used for the experiments of cell proliferation assay.

PBMC were separated by centrifugation of heparinized blood on Ficoll-Paque Plus. Ag-specific CD4-positive short-term T cell lines were generated from PBMC suspensions as previously described (25). Briefly, PBMC were stimulated in RPMI 1640 medium containing 5% autologous serum with streptokinase (100 U/ml) for 5 days. On day 6, activated T cells were expanded in the presence of human IL-2 (20 U/ml), and on day 15, they were used for migration assay. The expression of CXCR3 on the cells was identified by flow cytometry.

Trophoblast cells were prepared and maintained as previously described with some modifications (26–27). Briefly, the tissues were washed in PBS, and the soft villous material was cut away from connective tissue and vessels. The washed tissue was incubated in sterile PBS containing 1 mM $MgSO_4$, 0.125% trypsin, and 30 U/ml DNase I for 30 min at 37°C with mild stirring, the suspension was filtered through a 100-μm nylon cell strainer, and the cells were centrifuged at 200 × *g* for 5 min to obtain a cell pellet, which was resuspended in Medium 199 with 10% FBS. The suspension was layered onto Ficoll-Paque Plus and centrifuged at 150 × *g* for 15 min. Trophoblast cells recovered from the interface were washed with PBS and resuspended in Medium 199. The remaining leukocytes and syncytiotrophoblasts were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblasts. The cells were washed with PBS and the medium was changed to Medium 199 with 10% FBS and placed in a type IV collagen-coated 6-well plate (BD Biosciences) and kept at 37°C in a humidified 5% CO_2/95% air atmosphere. After incubation for 2 or 3 days, the cells were trypsinized and used for the experiments.

Treatment of cell cultures

When EEC and ESC approached confluence, the complete medium was removed and replaced with fresh medium and antibiotics, and the cells were cultured for an additional 12–24 h. To evaluate the dose effects of IFN-γ, wells were replenished with serum-free medium with different concentrations of IFN-γ and the cells were incubated for 24 h. To assess the effect of IFN-γ on the expression of CXCL11 mRNA in EEC and ESC, the cells were incubated with IFN-γ at different concentrations in serum-free medium for 6 h. After the treatments, the conditioned medium were collected, centrifuged, and stored at −80°C for subsequent analysis.

Immunocytochemistry

In 48 h of culture, EEC, ESC, and trophoblast cells were fixed in cold methanol/acetone (1:1) at −20°C for 20 min and were washed twice in PBS. The fixed cells were treated with 3% hydrogen peroxide for 5 min to eliminate endogenous peroxidase. After blocking with 1.5% horse serum for 20 min, the cells were incubated with mouse mAbs to cytokeratin, vimentin, cytokeratin-7, and CD45 for 30 min at room temperature. Control cells were incubated with nonimmune murine IgG1, the concentration of which was adjusted to that of the primary Ab. The cells were then incubated with biotinylated goat anti-mouse IgG, followed by incubation with peroxidase-labeled streptavidin solution for 20 min at room temperature. The chromogenic reaction was conducted with diaminobenzidine. All cells were counterstained with hematoxylin. The experiments were repeated four times.

Western blotting

Cultured cells were homogenized in the lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue and diluted to 1 mg of total protein/ml. The protein concentration in the homogenized cells was measured by a protein assay kit (Bio-Rad). Samples were resolved by 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit polyclonal Abs to human CXCR9 (1/1000), human CXCL10 (1/1000), human CXCL11 (1/1000), total p42/44 MAPK (1/1000), and phosphospecific p42/44 MAPK (1/1000) as primary Abs and anti-rabbit HRP Ab (1/1000) as a secondary Ab. Immune complexes were visualized by use of ECL Western blotting system (Amersham Biosciences).

Measurement of CXCL11 in the supernatants of culture media

Concentrations of CXCL11 in conditioned culture media were measured using its specific ELISA kit (Quantikine; R&D Systems) according to the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan Microplate Reader (ASYS Hitech). Cultured cells were homogenized and the total protein amount in the homogenized cells was measured by a protein assay kit. Data were standardized by total protein of cell lysates.

RNA extraction, reverse transcription (RT), and real-time quantitative PCR of CXCL11, CXCR3, and its spliced variant CXCR3-B mRNA

RT and real-time quantitative PCR were performed as we have reported previously (21–24, 28–30). Total RNA was extracted from EEC and ESC, using the RNAeasy Mini kit (Qiagen). RT was performed using Rever Tra Ace-α (Toyobo); real-time quantitative PCR and data analysis were performed using LightCycler (Roche Diagnostic), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a 20-μl total volume and cDNA was amplified using oligonucleotide primers based on human CXCL11, CXCR3, and the CXCR3-B sequence.

Recent studies indicate that in addition to the classic receptor CXCR3-A, alternatively spliced variant CXCR3-B is expressed in some cell types (31). CXCR3 primers amplified a common sequence to CXCR3-A and CXCR3-B. CXCR3-B primers amplified a unique sequence to CXCR3-B.

CXCL11 primers (sense, 5′-TTAAACAAACATGAGTGTGAAGGG-3′; antisense, 5′-CGTTGTCCTTTATTTTCTTTCAGG-3′) were chosen to amplify a 228-bp fragment. CXCR3 primers (sense, 5′-TGCCAATA CAACTTCCCACA-3′; antisense, 5′-CGGAACTTGACCCCTACAAA-3′) were chosen to amplify a 371-bp fragment. CXCR3-B primers (sense,

[2] Abbreviations used in this paper: EEC, endometrial epithelial cell; ESC, endometrial stromal cell; RT, reverse transcription; C_T, threshold cycle; LDH, lactate dehydrogenase; PI, propidium iodide.

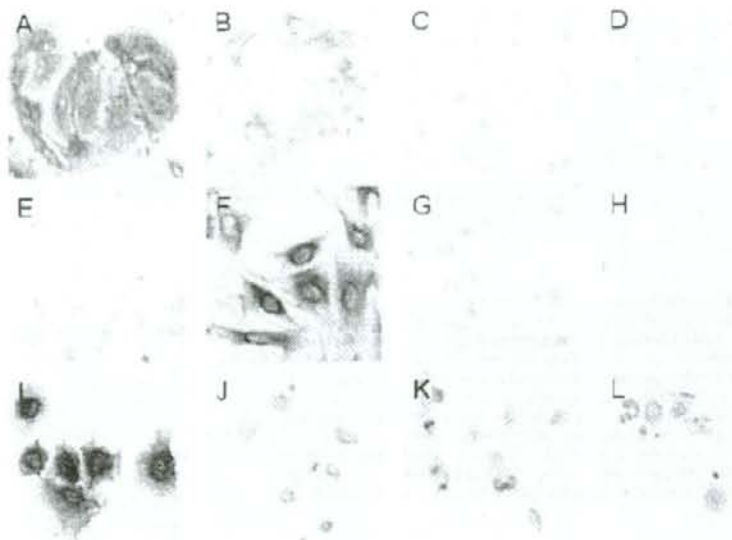

FIGURE 1. Immunocytochemical staining of primary cultured EEC, ESC, and trophoblast cells in early pregnancy. *A–D*, EEC were stained by anti-cytokeratin mAb (*A*), anti-vimentin mAb (*B*), anti-CD45 mAb (*C*), and isotype mIgG1 (*D*). EEC were positively stained with cytokeratin. *E–H*, ESC were stained by anti-cytokeratin mAb (*E*), anti-vimentin mAb (*F*), anti-CD45 mAb (*G*), and isotype mIgG1 (*H*). ESC were positively stained with vimentin. *I–L*, Trophoblast cells were stained by anti-cytokeratin-7 mAb (*I*), anti-vimentin mAb (*J*), anti-CD45 mAb (*K*), and isotype mIgG1 (*L*). Trophoblast cells were positively stained with cytokeratin-7. All cells were counterstained with hematoxylin. The result is representative of four separate experiments.

5′-TCACAAAAGAGTTCCTGCCA-3′; antisense, 5′-AAGAGGAGGCT GTAGAGGGC-3′) were chosen to amplify a 241-bp fragment. Expression of CXCL11, CXCR3, and CXCR3-B mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. Human GAPDH primers (Toyobo) were chosen to amplify a 452-bp fragment. The real-time PCR condition of CXCL11 was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 16 s, followed by melting curve analysis. The PCR condition of CXCR3 was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 15 s, followed by melting curve analysis. The PCR condition of CXCR3-B was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 10 s, followed by melting curve analysis. Standardization of the data was performed by subtracting the signal threshold cycles (C_T) of the internal standard (GAPDH) from the C_T of CXCL11, CXCR3, and CXCR3-B. To quantify the expression of CXCR3-A, we subtracted the amount of CXCR3-B from that of CXCR3. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen) and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Flow cytometric analysis

Flow cytometric analysis was performed as we have reported previously (22). Adherent cells (EEC, ESC, and trophoblast cells) were detached by using 0.25% trypsin/EDTA. The cells (2×10^5 cells/sample) were washed twice with PBS containing 2% FBS and stained with the mouse anti-human CXCR3 mAb or isotype control mouse IgG1 for 30 min on ice. Then, the cells were washed twice and incubated with FITC-conjugated anti-mouse IgG Ab for 30 min on ice. Being washed twice, the cells were analyzed using EPICS XL flow cytometer (Beckman-Coulter) and EXPO 32 software (Beckman Coulter).

Immunohistochemistry

Human endometrial and placental tissues were fixed overnight in 10% neutral-buffered formalin and embedded in paraffin, and 5-μm sections were prepared. Sections were treated with 0.03% hydrogen peroxide for 30 min to eliminate endogenous peroxidase. After blocking with 1.5% BSA, the sections were incubated with anti-human CXCR3 mAb (1/400) for 30 min at room temperature. Control slides were incubated with isotype control mouse IgG1, the concentration of which was adjusted to that of the primary Ab. The sections were then incubated with biotinylated goat anti-mouse IgG, followed by incubation with peroxidase-labeled streptavidin solution for 20 min at room temperature. The chromogenic reaction was conducted with diaminobenzidine. All sections were counterstained with hematoxylin. Assessments of immunostaining were based on agreement among three independent observers who were blind to the phases of the menstrual cycle at which specimens were collected.

In vitro migration assay

Migration assay was performed in 24-well plates (Costar) carrying Transwell permeable supports with 3-μm polycarbonate membrane for T cells and with 8-μm polycarbonate membrane for trophoblast cells as previously reported (32, 33). Supernatants of EEC were either stimulated or not by IFN-γ (100 ng/ml) for 24 h, were preincubated for 1 h with 10 μg/ml anti-CXCL9/10/11 Ab or isotype control rabbit IgG, and were plated on the lower chambers. Cells were plated on the upper wells of Transwell membranes containing 100 μl of serum-free DMEM/F12. A total of 5×10^6 T cells were incubated for 2 h at 37°C and 5% CO_2 atmosphere, and 2×10^5 trophoblast cells were for 24 h. T cells and trophoblast cells on the upper surface of membranes were completely removed; migrated cells were fixed with acetone/methanol. The number of T cells, resuspended in 10 ml PBS, was determined using a Coulter Counter Z1 (Beckman Coulter). Migration indices of trophoblast cells were determined by counting the number of trophoblast cells stained with H&E in 10 randomly selected nonoverlapping fields of the wells under light microscope.

Cell proliferation assay

Cell proliferation assay was performed as we have reported previously (21, 30, 34, 35). The effect of CXCL11 on the proliferation of ESC and EEC was examined by measuring BrdU incorporation into DNA by using the Biotrak Cell Proliferation ELISA System (Amersham Biosciences) according to the manufacturer's instructions. ESC and EEC were seeded into Falcon 96-multiwell plates (BD Biosciences) at a density of 1×10^4 cells/well in 100 μl of the culture medium. To assess the effect of CXCL11 on cell proliferation, the cells were incubated with CXCL11 at different concentrations in serum-free medium. To evaluate the effects of a MAPK inhibitor, the cells were preincubated with MEK inhibitor PD98059 for 1 h before CXCL11 treatment. After 24 h, 100 μl of BrdU solutions were added and incubated at 37°C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA was denatured by the addition of 200 μl/well fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction and the resultant color was read at 450 nm in the DigiScan Microplate Reader.

Assessment of cell death

Cytotoxicity of EEC was assessed by the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into supernatant using the Cytotoxicity Detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Absorbance was read at 492 nm with the DigiScan Microplate Reader. Values were expressed relative to measurement from control LDH.

Table 1. *IFN-γ-stimulated CXCL11 mRNA expression in EEC and ESC*[a]

	IFN-γ (ng/ml)			
	0 (=control)	10	100	1000
EEC	0.0028 ± 0.0020	0.21 ± 0.15[b]	0.74 ± 0.41[b]	0.96 ± 0.72[b]
ESC	0.00039 ± 0.00029	0.23 ± 0.21[c]	0.37 ± 0.30[c]	0.41 ± 0.32[c]

[a] The values of CXCL11 mRNA represent relative ratios compared with GAPDH mRNA level. Values are the mean ± SEM of three separate experiments using different EEC and ESC preparations. Logarithm of the values was used in statistical analysis.
[b] $p < 0.0001$, each vs. control of EEC.
[c] $p < 0.0001$, each vs. control of ESC.

Apoptosis of EEC was assessed by double staining of annexin V and propidium iodide (PI) using an Annexin V[FITC] kit (Beckman Coulter) according to the manufacturer's instructions. Annexin V is a phosphatidylserine-binding protein used to detect phosphatidylserine translocation from the inner to the outer plasma membrane leaflet which is assumed to be a feature of apoptosis. Cell death, including necrosis and late phase of apoptosis, was detected by PI, a marker for cell membrane permeability. Briefly, EEC were detached by using 0.25% trypsin/EDTA, washed twice with PBS, and pelleted in annexin V-binding buffer containing FITC-conjugated annexin V. PI was then added and samples were incubated for 10 min on ice and analyzed by EPICS XL flow cytometer and EXPO 32 software.

Statistical analysis

Data were evaluated using ANOVA with posthoc analysis (Fisher's protected least significance) for multiple comparisons and the Student *t* test for paired comparisons. A value of $p < 0.05$ was accepted as significant.

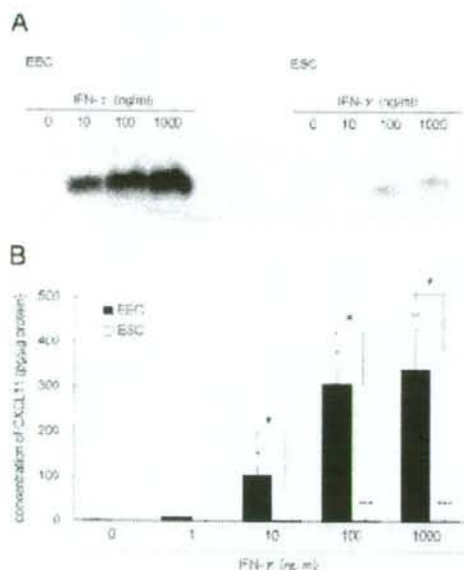

FIGURE 2. IFN-γ-stimulated CXCL11 protein production in EEC and ESC. *A*, EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. Cell extracts were prepared and assayed for CXCL11 by Western blotting. The result is representative of three separate experiments. *B*, EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. The conditioned medium were collected and assayed for CXCL11 concentrations by ELISA. The values were normalized with total protein of cell extracts. Values are the mean ± SEM of the combined data of five separate experiments using different EEC and ESC preparations. #, $p < 0.0001$, between EEC and ESC with IFN-γ at 10, 100, 1000 ng/ml. *, $p < 0.01$; **, $p < 0.0001$, both vs control of EEC; ***, $p < 0.0005$, both vs control of ESC.

Results

Verification of the purity of EEC, ESC, and trophoblast cells

We confirmed the purity of EEC, ESC, and trophoblast cells with immunocytochemistry. The purity of EEC preparations was >95%, as judged by positive cellular staining for cytokeratin and negative cellular staining for vimentin and CD45 (Fig. 1, *A–D*). The purity of ESC preparations was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45 (Fig. 1, *E–H*). The purity of trophoblast cell preparations was >90%, as judged by positive cellular staining for cytokeratin-7 and negative staining for vimentin and CD45 (Fig. 1, *I–L*).

IFN-γ-induced protein and mRNA expression of CXCL11 in EEC and ESC

IFN-γ stimulated the mRNA expression and the cellular and secreted protein levels of CXCL11 in a dose-dependent manner in EEC and ESC (Table I and Fig. 2). CXCL11 protein levels both in the cells and in the medium were remarkably higher in EEC than in ESC. The secreted protein levels of CXCL11 in the control were 1.41 ± 0.26 pg/μg protein in EEC, but undetectable in ESC. IFN-γ at 100 ng/ml increased secreted CXCL11 protein levels up to 308 ± 35 pg/μg protein in EEC and 3.16 ± 1.69 pg/μg protein in ESC.

IFN-γ-induced protein expression of three CXCR3 ligands CXCL9, CXCL10, and CXCL11 in EEC

As illustrated in Fig. 3, IFN-γ dose-dependently induced CXCL9, CXCL10, and CXCL11 in EEC.

Expression of CXCR3 in EEC, ESC, and trophoblast cells

We examined the expression of CXCR3 in cultured EEC, ESC, and trophoblast cells. As a positive control, activated T cells showing a Th1-polarized profile of cytokine production were used

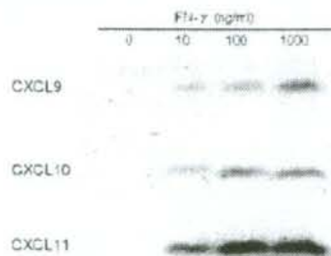

FIGURE 3. IFN-γ-induced protein expression of three CXCR3 ligands CXCL9, CXCL10, and CXCL11 in EEC. EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. Cell extracts were prepared and assayed for CXCL9, CXCL10, and CXCL11 by Western blotting. All the chemokines were expressed in dose-dependent manners. The result is representative of three separate experiments.

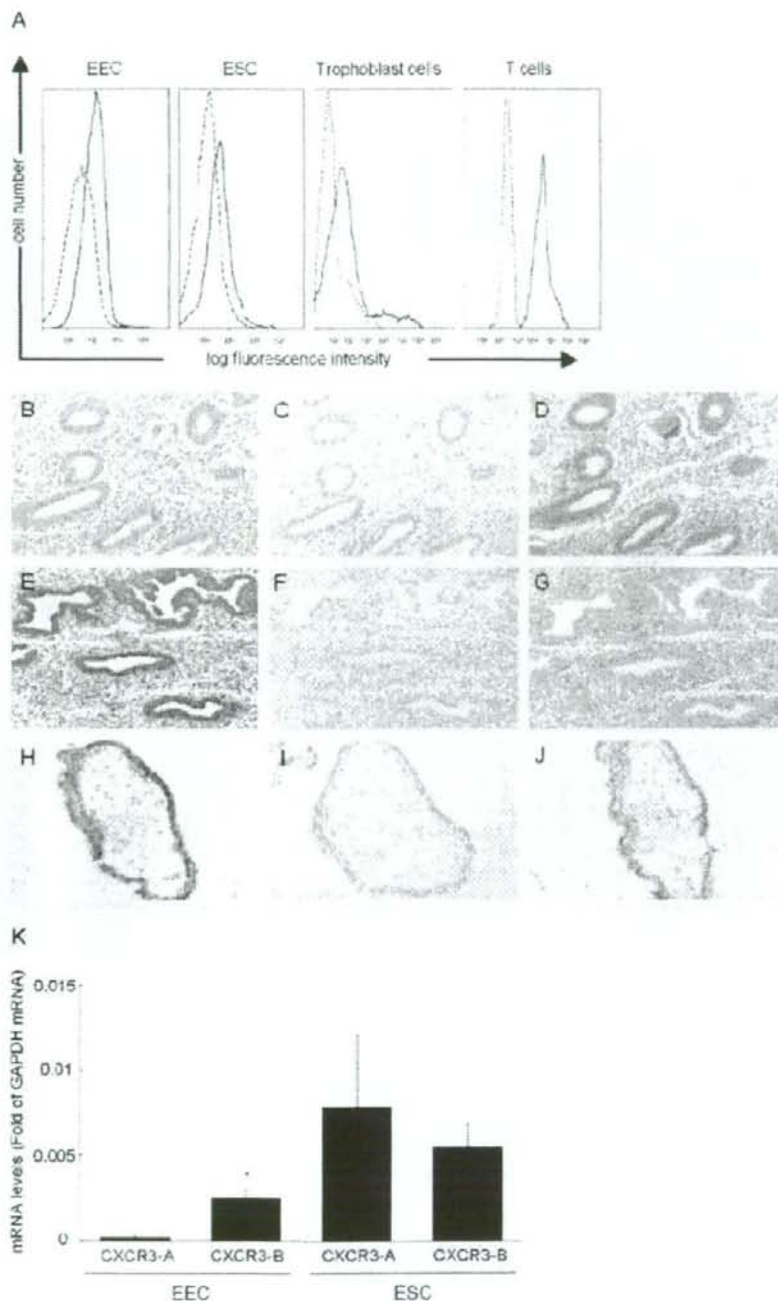

FIGURE 4. Expression of CXCR3 in human EEC, ESC, and trophoblast cells. *A*, Cultured ESC, EEC, and trophoblast cells were collected and stained with anti-CXCR3 Ab (solid line) or isotype control mouse IgG1 (dotted line). T cells were used to be positive control cells for CXCR3. *B–G*, Immunohistochemistry of CXCR3 in human endometrium. Endometrial sections of proliferative phase (*B–D*) and secretory phase (*E–G*) were immunostained with anti-human CXCR3 mouse Ab (*B* and *E*) or isotype mouse IgG1 (*C* and *F*) and stained with H&E (*D* and *G*). Magnification, ×200. *H–J*, Immunohistochemistry of CXCR3 in human villi. Sections of villi were immunostained with anti-human CXCR3 mouse Ab (*H*) or isotype mouse IgG1 (*I*) and stained with H&E (*J*). Magnification, ×200. *K*, Expression of CXCR3-A and -B mRNA in EEC and ESC. Total RNA isolated from EEC and ESC of 13 women was reverse transcribed and amplified by PCR using primers of CXCR3 and CXCR3-B. CXCR3 primers amplify common sequence to CXCR3-A and -B. The data were calculated by subtracting the signal C_T of the internal standard (GAPDH) from the C_T of CXCR3 and CXCR3-B. To quantify the expression of CXCR3-A, we subtracted the amount of CXCR3-B from that of CXCR3. The values of CXCR3-A and -B mRNA represent relative ratios compared with GAPDH mRNA level. Values are the mean ± SEM of samples from 13 women. *, $p < 0.0001$, CXCR3-A mRNA in EEC vs CXCR3-B mRNA in EEC.

(36, 37). As shown in Fig. 4*A*, flow cytometry using an anti-CXCR3 Ab demonstrated that all the four types of cells, i.e., EEC, ESC, trophoblast cells, and Th1 cells, expressed CXCR3 on the cell surface.

As shown in Fig. 4, *B–G*, the presence of CXCR3 in human endometrium was demonstrated in both proliferative and secretory phases. Both stromal and epithelial cells were stained. The intensity of staining in EEC appeared to be stronger than that in ESC in the same section, regardless of the phases of the menstrual cycle. The intensity of the staining seems relatively weak during the proliferative phase and was enhanced during the secretory phase. The presence of CXCR3 in human villi was shown in Fig. 4, *H–J*.

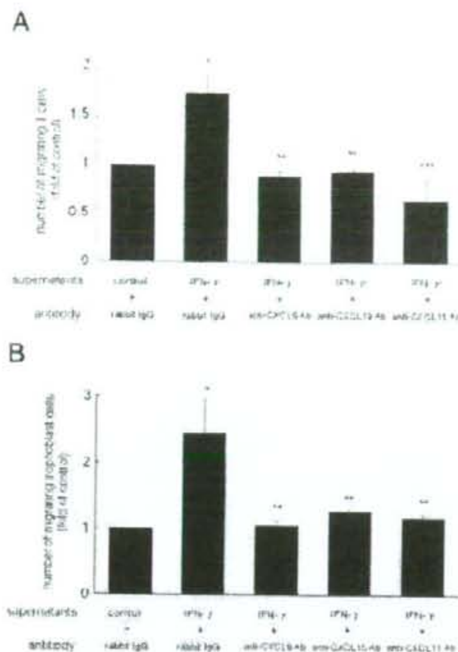

FIGURE 5. Effects of conditioned medium of IFN-γ-stimulated EEC on the migration of T cells and trophoblast cells. Migration assay was performed to study whether the migration of T cells and trophoblast cells was affected by endometrial CXCL11 expression. Supernatants of EEC either stimulated or not by IFN-γ (100 ng/ml) for 24 h were preincubated for 1 h with 10 μg/ml anti-CXCL9 Ab, anti-CXCL10 Ab, anti-CXCL11 Ab, or isotype control rabbit IgG, and plated on the lower chambers. Cells were plated on the upper wells of Transwell membranes containing 100 μl of serum-free DMEM/F12. 5×10^6 T cells (*A*) were incubated for 2 h, and 2×10^5 trophoblast cells (*B*) were for 24 h. After the incubation, T cells and trophoblast cells on the upper surface of membranes were completely removed and migrated cells were fixed with acetone/methanol. Migration indices were determined by counting the cell number. The values represent relative ratios of the cell number compared with those in using the control supernatants of EEC with rabbit IgG. *A*, Values are the mean ± SEM of the combined data from three independent experiments using different T cell preparations. *, $p < 0.05$, control with rabbit IgG vs IFN-γ with rabbit IgG. **, $p < 0.001$, each vs IFN-γ with rabbit IgG. *B*, Values are the mean ± SEM of the combined data from three independent experiments using different trophoblast cell preparations. *, $p < 0.05$, control plus rabbit IgG vs IFN-γ plus rabbit IgG. **, $p < 0.05$, each vs IFN-γ plus rabbit IgG.

Trophoblast cells were strongly stained. No staining was seen when the primary Ab was replaced with nonimmune mouse IgG1.

Expression of CXCR3-A and CXCR3-B mRNAs in EEC and ESC

We examined the mRNA expression of CXCR3-spliced variants CXCR3-A and CXCR3-B in cultured EEC and ESC. In EEC, expression level of CXCR3-B mRNA was 12 times as high as that of CXCR3-A, whereas, in ESC, there was no significant difference between expression levels of CXCR3-A and CXCR3-B (Fig. 4*K*).

Stimulation of IFN-γ on the migration of T cells and trophoblast cells through secretion of CXCR3 ligands

To study chemotactic activity of CXCR3 ligands secreted from EEC including CXCL11 on the migration of T cells and trophoblast cells, in vitro migration assay was performed. As illustrated

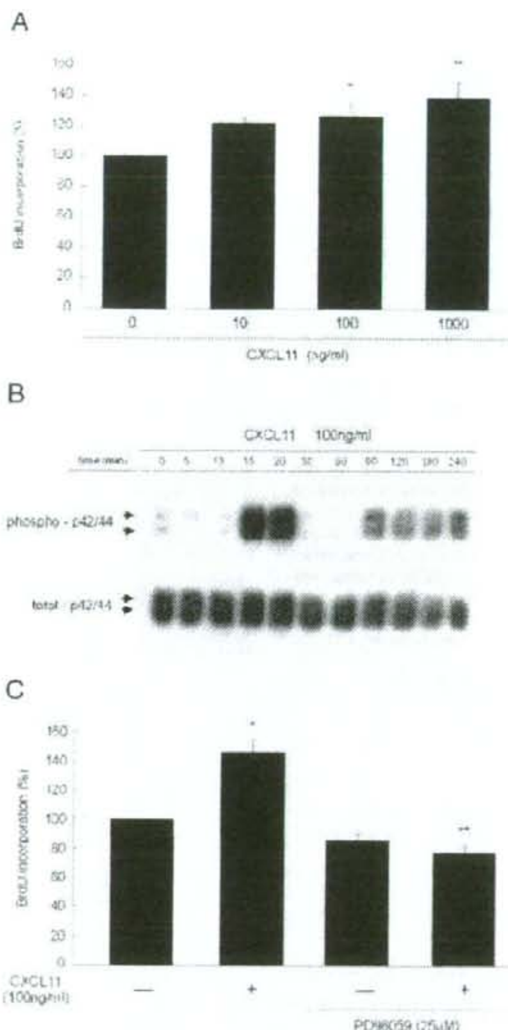

FIGURE 6. CXCL11-induced ESC proliferation via p42/44 MAPK activation. *A*, The effect of CXCL11 on the proliferation of ESC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. ESC were treated with CXCL11 at different concentrations for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from five independent experiments using different ESC preparations. *, $p < 0.005$; **, $p < 0.0005$, both vs control. *B*, ESC were incubated with 100 ng/ml CXCL11 for the indicated times (0–240 min). Cell extracts were prepared and assayed for phosphorylated p42/44 MAPK (phospho-p42/44) or total p42/44 MAPK (total-p42/44) by Western blotting. The result is representative of three separate experiments. *C*, Effects of MEK inhibitor PD98059 on CXCL11-induced cell proliferation of ESC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. ESC were treated with or without PD98059 (25 μM), for 1 h, and then stimulated with CXCL11 (100 ng/ml). After 24 h incubation, BrdU incorporation into DNA in ESC was measured using the cell proliferation ELISA. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from four independent experiments using different ESC preparations. *, $p < 0.0001$, control vs CXCL11. **, $p < 0.0001$ CXCL11 vs CXCL11 with PD98059.

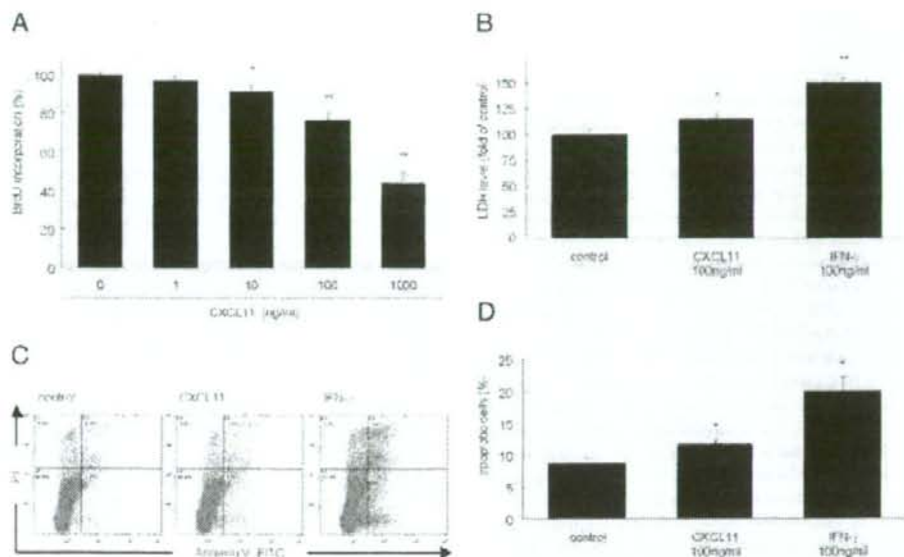

FIGURE 7. CXCL11-induced inhibition of proliferation and stimulation of apoptosis in EEC. *A*, The effect of CXCL11 on the proliferation of EEC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. EEC were treated with CXCL11 at different concentrations for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from four independent experiments using different EEC preparations. *, $p < 0.05$; **, $p < 0.0001$, both vs control. *B*, The effect of CXCL11 on cell death of EEC was determined by the measurement of LDH in CXCL11-treated EEC supernatant. EEC were treated with CXCL11 at 100 ng/ml or with IFN-γ at 100 ng/ml for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from three independent experiments using different EEC preparations. *, $p < 0.05$; **, $p < 0.0001$, both vs control. *C*, The effect of CXCL11 on apoptosis of EEC was determined by double staining of annexin V and PI. EEC were treated with CXCL11 at 100 ng/ml or with IFN-γ at 100 ng/ml for 48 h. The cells were stained with Annexin V^{FITC} and PI. Apoptosis was analyzed by flow cytometry on 5×10^4 EEC. The result is representative of four separate experiments. *D*, The percentage of apoptotic EEC treated with CXCL11 and IFN-γ was significantly higher than that of the control. Annexin V-positive cells were regarded as apoptotic cells. Values are the mean ± SEM of the combined data from four independent experiments using different EEC preparations. *, $p < 0.05$, both vs control.

in Fig. 5, supernatants of IFN-γ-stimulated EEC up-regulated the migration of T cells and trophoblast cells as compared with those of nonstimulated EEC. Moreover, immunoneutralization with Abs of three CXCR3 ligands, CXCL9, CXCL10, and CXCL11, reduced the chemotactic activity of IFN-γ-stimulated supernatants.

CXCL11-induced proliferation of ESC via the p42/44 MAPK pathway

Cell proliferative effects of CXCL11 on EEC were studied by BrdU incorporation assay. As shown in Fig. 6*A*, BrdU incorporation into DNA was significantly increased by CXCL11 at 100–1000 ng/ml. At the concentration of 100 ng/ml, the level of BrdU incorporation was 127% of the control.

It has been reported that CXCR3 ligands induce activation of p42/44 MAPK and cell proliferation in vascular pericytes, glomerular mesangial cells, and that the mitogenic response is mediated by p42/44 MAPK signaling (38). We therefore tested whether activation of p42/44 MAPK was required for cell proliferation induced by CXCL11 in ESC. As depicted in Fig. 6*B*, CXCL11 at 100 ng/ml stimulated a biphasic phosphorylation of p42/44 MAPK in ESC. The phosphorylation levels were reached maximal at 15 and 20 min, followed by decrease to basal levels in 30–60 min and reincrease over 90 min. A p42/44 MAPK pathway inhibitor (MEK inhibitor) PD98059 significantly abrogated the CXCL11-induced BrdU incorporation of ESC (Fig. 6*C*).

CXCL11 inhibited proliferation and stimulated apoptosis in EEC

In contrast to ESC, EEC showed significantly decreased BrdU incorporation into DNA by addition of CXCL11 at 10–1000 ng/ml (Fig. 7*A*). At the concentration of 100 ng/ml, the level of BrdU incorporation was down to 77% of the control.

Effects of CXCL11 on cell death of EEC were determined by measurement of LDH in the supernatants. As shown in Fig. 7*B*, the addition of CXCL11 increased the release of LDH from EEC significantly. Additions of 100 ng/ml CXCL11 and 100 ng/ml IFN-γ enhanced the levels of LDH up to 115 and 150% of the control, respectively.

Apoptotic effects of CXCL11 on EEC were evaluated by staining of annexin V. Fig. 7*C* shows a representative data. Cells expressing annexin V (*lower* and *upper right quadrants* combined) were defined as apoptotic dells. The percentage of apoptotic cells in CXCL11- and IFN-γ-stimulated cells was higher than that in the control cells. As shown in Fig. 7*D*, the combined data from four independent experiments demonstrated that apoptotic cells were significantly increased by CXCL11 at 100 ng/ml.

Discussion

In the present study, we demonstrated that IFN-γ induced production of the chemokine CXCL11 in EEC and that the receptor of CXCL11, CXCR3, is expressed in EEC, ESC, and trophoblast cells. CXCL11 secreted from EEC stimulated migration of trophoblast cells

and T cells. In addition, CXCL11 stimulated proliferation of ESC and apoptosis of EEC.

The present finding that IFN-γ induced the production of CXCL11 in EEC in a markedly larger amount than in ESC implies an endometrial response to the adjacent embryo in apposition and attachment, an initial phase of implantation. IFN-γ production from the human embryo is highest when it develops a blastocyst and reaches a point of apposition in the uterus (8), suggesting that IFN-γ have regulatory roles at the beginning of implantation of the embryo. The endometrial epithelium is an important element where the molecular interactions between the embryo and the endometrium commence (2–4). Therefore, it is feasible that embryo-derived IFN-γ play important roles for implantation partly by provoking CXCL11 production in EEC.

CXCL11 had a positive proliferative effect on ESC and a negative effect on EEC, while both cells have CXCR3, a receptor of CXCL11. It has been reported that CXCR3 ligands, CXCL9, CXCL10, and CXCL11, up- or down-regulate cell proliferation in a cell type-dependent manner. For example, they stimulate proliferation of human vascular pericytes, including glomerular mesangial cells (38, 39) and smooth muscle cells (40), whereas they inhibit growth of vascular endothelial cells. It is interesting to note that the reverse response pattern demonstrated in ESC and EEC mimics that observed in vascular pericytes and endothelial cells. In a recent study, CXCR3-B, when activated by its ligands, induces apoptosis and inhibits cell proliferation (31). In contrast, activated CXCR3-A induces cell proliferation (31). The present study demonstrated that CXCR3-B was mainly expressed in EEC and both CXCR3-A and -B were expressed in ESC. The opposite functions of CXCL11 on cell proliferation might be explained by the interaction of CXCL11 with CXCR3 variants.

The present study showed that CXCL11 induced a biphasic activation of p42/44 MAPK. Interaction between CXCR3 and its ligands leads to p42/44 MAPK activation, whose pattern is either monophasic or biphasic (38). A biphasic activation of p42/44 MAPK has been indicated to stimulate progression of the cell cycles (41, 42). It is thus speculated that CXCL11 promotes ESC proliferation through a biphasic activation of p42/44 MAPK.

Cell death induced by CXCL11 may play a physiological role in the process of implantation. Cell death, especially apoptosis, of endometrial epithelial cells occurs in implantation sites not only in mice (43), rats (44), and hamsters (45), but also in humans (3, 46). Embryo-induced apoptosis of epithelial cells is an important mechanism for invading the luminal epithelium and breaching the epithelial barrier; the immediate consequence is that the trophoectoderm come in direct contact with the basement membrane and, then, stromal invasion can proceed (3). The apoptotic mechanism in endometrial epithelial cells is triggered by a direct contact between blastocysts and epithelial cells (3). In view of the present finding that IFN-γ and CXCL11 induced apoptosis of EEC, we speculate that embryo-derived IFN-γ kills EEC for implantation and that the apoptotic effect is partially indebted to IFN-γ-induced CXCL11 in EEC.

A chemotactic activity of CXCR3 ligands, including CXCL11, on the trophoblast may subserve spreading and invasion of trophoblast cells during the implantation period. Multiple factors such as insulin-like growth factor II, insulin-like growth factor-binding protein-1, endothelin-1, and heparin-binding epidermal growth factor have been shown to promote migration of trophoblast cells into the endometrium (47). In an ovine study, CXCL10 expressed in the endometrium was suggested to stimulate the migration and attachment of trophoblast cells (19). The present study demonstrated that CXCR3 ligands, CXCL9, CXCL10, and CXCL11, which derived from EEC stimulated by IFN-γ, increased migration of trophoblast cells as well as T cells. CXCR3 expressed on the trophoblast cells, which was demonstrated by immunohistochemistry and flow cytometry, may be involved in the chemotactic effect of CXCR3 ligands. Taken together, CXCR3 ligands including CXCL11 could be added to the list of chemotactic factors of trophoblast cells.

Several chemokines expressed in the fetal and maternal annexes during the early pregnancy are thought to regulate cellular movement and positioning of leukocytes, which infiltrated into the subepithelial stromal regions of the uterus. CXCL9 and CXCL10 are suspected to modulate the distribution of leukocytes in the endometrium, contributing to the establishment of immunological environments suitable for implantation and subsequent development (17). Chemotactic activity of three CXCR3 ligands on T cells may also tune the immune environments of the endometrium for implantation.

In summary, we have shown that IFN-γ induced expression of CXCL11 in the endometrial epithelial cells and that CXCR3, the receptor of CXCL11, is expressed on the endometrial cells and trophoblast cells. The demonstrated pleiotropic functions of CXCL11 on trophoblast cells and endometrial cells are suggested to regulate the implantation process of the embryo.

Disclosures

The authors have no financial conflict of interest.

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Recurrence of ovarian endometrioma after laparoscopic excision

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BACKGROUND: To analyse risk factors that influence the recurrence of endometrioma after laparoscopic excision. METHODS: A total of 224 patients who had a minimum of 2 years of post-operative follow-up after laparoscopic ovarian endometrioma excision were studied retrospectively. Recurrence was defined as the presence of endometrioma more than 2 cm in size, detected by ultrasonography within 2 years of surgery. Fourteen variables (age, presence of infertility, pain, uterine myoma, adenomyosis, previous medical treatment of endometriosis, previous surgery for ovarian endometriosis, single or multiple cysts, the size of the largest cyst at laparoscopy, unilateral or bilateral involvement, co-existence of deep endometriosis, revised American Society for Reproductive Medicine (ASRM) score, post-operative medical treatment and post-operative pregnancy) were evaluated to assess their independent effects on the recurrence using logistic regression analysis. RESULTS: The overall rate of recurrence was 30.4% (68/224). Significant factors that were independently associated with higher recurrence were previous medical treatment of endometriosis [odds ratio (OR) = 2.324, 95% confidence interval (95% CI) = 1.232–4.383, P = 0.0092) and larger diameter of the largest cyst (OR = 1.182, 95% CI = 1.004–1.391, P = 0.0442). Post-operative pregnancy was associated with lower recurrence (OR = 0.292, 95% CI = 0.028–0.317, P = 0.0181). CONCLUSIONS: Previous medical treatment of endometriosis or large cyst size was a significant factor that was associated with higher recurrence of the disease. Post-operative pregnancy is a favourable prognostic factor.

Key words: endometriosis/laparoscopy/ovary/recurrence/risk factors

Introduction

Ovarian endometrioma is a common disease lesion among women with endometriosis. Regardless of its symptoms, surgery is most frequently chosen for its treatment because medical treatment alone is inadequate (Jones and Sutton, 2000). In addition, a likelihood of malignant change in this disease is not negligible (Nishida *et al.*, 2000), and European Society of Human Reproduction and Embryology (ESHRE) guidelines recommend that histology should be obtained to exclude malignancy in cases of endometrioma of more than 3 cm in diameter (Kennedy *et al.*, 2005).

Because this disorder is commonly diagnosed in women of reproductive age (Giudice and Kao, 2004), laparoscopic excision of endometrioma, instead of oophorectomy, is applied for most cases. When it is done in infertile woman, laparoscopic excision is also known to improve fertility (Beretta *et al.*, 1998).

One of the most frustrating aspects of treating endometrioma with laparoscopic excision is disease recurrence after surgery (Busacca *et al.*, 1999). When planning a laparoscopy, gynaecologists should be aware of each individual's expected likelihood of recurrence as well as her symptoms and desire for current or future fertility. By having information about factors that may be related to a recurrence of ovarian endometrioma, gynaecologists will be able to distinguish patients at risk, optimize the timing of laparoscopy and plan pre- and post-operative management properly. However, little study has been done to analyse various variants that may have impacts on a recurrence of endometrioma after laparoscopic excision.

To date, recurrence of ovarian endometrioma after laparoscopy has always been discussed focusing on a single factor, such as the effect of post-operative (Muzii *et al.*, 2000) or pre-operative (Muzii *et al.*, 1996) medication, the method of laparoscopic treatment (Saleh and Tulandi, 1999) and the anatomical location (Ghezzi *et al.*, 2001). There is only one multivariate analysis that analysed six variables on the recurrence of

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