Altered decidual leucocyte populations in the placental bed in pre-eclampsia and foetal growth restriction: a comparison with late normal pregnancy

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Abstract

Alterations in the balance of leucocyte populations in uterine decidua may lead to the generation of an unfavourable cytokine environment that is associated with unsuccessful pregnancy. Single and double immunohistochemical labelling was used to examine leucocyte populations in decidua from normal third trimester, foetal growth-restricted and pre-eclamptic pregnancies. Placental bed biopsies from 12 women undergoing elective Caesarean section with no hypertension or foetal growth restriction (FGR), 8 women with FGR without maternal hypertension and 12 women with pre-eclampsia (PE) were used to quantify decidual CD56+ uterine NK cells, CD14+ macrophages, CD3+T-lymphocytes and CD8+ lymphocytes. CD3+CD56+, CD8+CD56+ and CD161+CD3+ double-labelled cells in decidua were compared in PE and control decidua. Decidual CD3+T-lymphocytes ($P<0.01$), CD8+ cytotoxic T-lymphocytes ($P<0.05$), CD14+ macrophages ($P<0.0001$) and CD56+ uterine natural killer (uNK) cells ($P=0.01$) were decreased in placental bed biopsies from women with PE compared with control third trimester decidua. By contrast, only CD56+ uNK cells were decreased in FGR decidua ($P<0.05$). Double-positive CD8+CD56+ cells were also decreased in PE compared with control third trimester decidua ($P<0.05$). The reduction in specific leucocyte subset numbers in PE and uNK cells in FGR suggests that altered local cytokine balance may be important in defective trophoblast invasion and spiral artery transformation in these pathological pregnancies.

Reproduction (2009) **138** 177–184

Introduction

Following implantation, the decidualised endometrium, a tissue containing a large population of leucocytes (King & Loke 1991, Stallmach *et al.* 1995), is rapidly infiltrated by foetally derived extravillous trophoblast cells (EVT; Kurman *et al.* 1984, Yeh *et al.* 1991), which express the paternally derived non-self HLA antigens, HLA-G, HLA-E and HLA-C (Houlihan *et al.* 1995, Hutter *et al.* 1996, King *et al.* 1996). In normal pregnancy, ~30–40% of decidual stromal cells are leucocytes (Bulmer *et al.* 1991); in the first trimester, uterine natural killer (uNK) cells account for up to 70%, macrophages up to 30% (Bulmer *et al.* 1988) and T-lymphocytes fewer than 20% (Bulmer *et al.* 1991) of the total leucocyte population (Bulmer *et al.* 1991, King & Loke 1991, Klentzeris *et al.* 1992). In contrast to first trimester decidua, third trimester decidua has been the subject of less research. Haller *et al.* (1993) reported an increase in CD3+ cells and a decrease in CD56+ (also known as NCAM1+) cells in third trimester compared with first

trimester decidua, while Vargas *et al.* (1993) found an increase in CD16+ lymphocytes at term, contrasting with low CD16+ cell numbers in first trimester decidua.

The large number of uNK cells found in early pregnant decidua may reflect a role in spiral artery remodelling. Following implantation, trophoblast cells invade the lumen and wall of the spiral arteries, replacing the musculoelastic media of the arterial wall with a fibrinoid matrix and leading to increased placental blood flow (Pijnenborg *et al.* 1981). Spiral artery remodelling has previously been considered to be a trophoblast-dependent process, but studies of human decidua have suggested that at least some remodelling precedes and occurs independently of interactions with EVT (Craven *et al.* 1998, Pijnenborg *et al.* 2006). In mice, this trophoblast-independent mechanism appears to be mediated by uNK cells (Ashkar *et al.* 2000); reconstitution with bone marrow from severe combined immunodeficient mice (deficient in T and B cells) largely corrected the spiral artery defects and decidual abnormalities (Guimond *et al.* 1998), implicating uNK

deficiency as the primary cause of the pathology. We have recently reported that uNK cells are a prominent source of angiogenic growth factors in the placental bed during early human pregnancy (Lash *et al.* 2006a), supporting the suggestion that uNK cells play a role in spiral artery remodelling.

Failure of trophoblast invasion and spiral artery remodelling has been associated with the development of both pre-eclampsia (PE) and foetal growth restriction (FGR) in the absence of maternal hypertension (Khong *et al.* 1986, Pijnenborg *et al.* 1991). In PE, reduced placental perfusion is associated with endothelial dysfunction and FGR leading to significant maternal and perinatal morbidity and mortality. Studies of human decidual leucocyte populations in PE and FGR are limited. Khong (1987) reported no differences in placental bed and non-placental bed leucocyte populations in PE compared with normal-term pregnant controls while Stallmach *et al.* (1999) reported an increase in CD8+ T cells and CD56+ uNK cells in decidua from women with PE. These observations were confirmed in a flow cytometric study, CD56+ cells also showing an altered phenotype with a shift from the CD16–CD56+ to the more cytotoxic CD16+CD56+ phenotype (Wilczynski *et al.* 2003). By contrast, Eide *et al.* (2006) noted a reduction in the proportion of leucocytes accounted for by CD56+ NK cells in decidua from pregnancies complicated with FGR with or without PE compared with control samples. Increased macrophage numbers both around and within myometrial spiral arteries and in the superficial decidua have been reported in pre-eclamptic women (Reister *et al.* 1999, Lockwood *et al.* 2006), leading to the suggestion that macrophage-induced apoptosis may be responsible for preventing EVT invasion of the spiral arteries (Reister *et al.* 2001). However, other groups have reported no differences or even reduced decidual macrophage numbers in PE (Burk *et al.* 2001, Redline 2001, Kim *et al.* 2007). Overall, therefore, the precise composition of decidual leucocytes in pregnancies complicated by PE and FGR remains controversial and unclear. This may in part be due to the different sources of decidual tissue investigated in these studies either obtained by vacuum suction of the placental bed after delivery (Eide *et al.* 2006), attached to the placenta delivered by Caesarean section (Burk *et al.* 2001, Wilczynski *et al.* 2003, Lockwood *et al.* 2006) or from placental bed biopsies (Reister *et al.* 1999, Stallmach *et al.* 1999, Kim *et al.* 2007).

The aim of this study was to use single and double immunohistochemical labelling to quantify and compare the major leucocyte populations (CD3+, CD8+, CD14+ and CD56+) in placental bed decidua from normal third trimester pregnancy and pregnancies complicated with PE or FGR.

Results

Pre-eclampsia

Single immunohistochemical labelling

The three major leucocyte populations identified in normal third trimester decidua were also present in pregnancies complicated by PE. The numbers of all cell types were reduced in PE compared with third trimester controls (Figs 1 and 2); CD3: PE 20.4 ± 1.9 versus controls 40.3 ± 6.7 ; $P < 0.01$; CD56: 18.3 ± 1.9 vs 29.0 ± 3.3 ; $P = 0.01$; CD14: 8.3 ± 0.7 vs 17.3 ± 1.2 ; $P < 0.0001$. In common with the results for CD3, the numbers of CD8+ lymphocytes were also reduced in PE compared with third trimester controls (19.4 ± 2.6 vs 34.8 ± 5.5 ; $P < 0.05$).

Double immunohistochemical labelling

Small populations of CD3+CD56+, CD161+ (also known as KLRB1C+) CD3+ and CD8+CD56+ cells were identified in both control third trimester pregnancy and PE decidua. No differences were found in the CD3+CD56+ and CD161+CD3+ cell populations between the two study groups (Table 1). By contrast, numbers of CD8+CD56+ double-labelled cells were reduced in PE (0.8 ± 0.3) compared with control third trimester samples (2.6 ± 0.90 , $P < 0.05$).

Foetal growth restriction

In FGR without maternal hypertension, there was a similar reduction in the numbers of the three main leucocyte types (Fig. 1; CD3: 24.0 ± 2.0 ; CD56: 14.1 ± 2.7 ; CD14: 12.0 ± 0.5) compared with control decidua. This reduction was, however, only significant for CD56+ uNK cells ($P < 0.05$), reductions in CD3+ ($P = 0.07$) and CD14+ ($P = 0.06$) just failing to reach statistical significance.

No significant differences were identified between decidual leucocyte populations in PE and FGR.

Figure 1 Decidual leucocyte numbers in FGR, PE and normal third trimester pregnancies. Data are expressed as mean cell count per $\times 250$ field and s.e.m. of multiple observations. * $P < 0.05$, ** $P < 0.001$.

Figure 2 Photomicrograph of control third trimester (a, c and e) and PE (b, d and f) placental bed decidua immunostained using an avidin–biotin peroxidase technique for CD3 (a and b) CD8 (c and d) and CD56 (e and f). Positive cells appear brown. Note reduction in all cell populations in pre-eclampsia compared with control third trimester samples. Magnification $\times 200$.

Discussion

Characterisation of decidual leucocyte populations in pregnancies complicated by PE or FGR may allow insights into the pathogenesis of these disorders. In the present study, all leucocyte populations present in decidua from women with PE or FGR. However, in decidua from women with PE, T lymphocytes, uNK cells and macrophages were all reduced in number, while in pregnancies complicated by FGR only the reduction in uNK cell numbers was statistically significant.

The altered leucocyte subset cell numbers observed in PE compared with third trimester controls may reflect

either a reduction in leucocyte infiltration or perturbed leucocyte composition. However, the relative proportion of each of the leucocyte subset cell populations did not alter between either PE or FGR and third trimester controls (control: 33% CD56, 47% CD3 and 20% CD14; PE: 38% CD56, 44% CD3 and 18% CD14; FGR: 28% CD56, 48% CD3 and 24% CD14). Therefore, the most likely explanation for the reduced leucocyte subset cell numbers noted in PE in the current study is that there is an overall reduction in leucocyte infiltration into the decidua of these women. Whether the decrease in decidual leucocytes is a cause or effect of PE cannot be determined.

The results indicate that there may be a common pathogenesis between PE and FGR, and this is the work that needs further investigation. The decreased numbers of CD56+ cells in both PE and FGR may be involved in decreased vascular adaptation to pregnancy during placentation. The lack of significant differences between control and FGR for CD3 and CD14 positive cells may reflect the smaller subject group of FGR, as numbers of these cells showed a trend for reduction. A possible limitation of the study is that placental bed biopsies sample only a small area of the placental bed and may not be representative of the whole area. The distribution of leucocytes in both PE and controls, however, were consistent and provided no evidence for local variations.

In contrast to the decidual leucocyte composition in early normal pregnancy (Bulmer *et al.* 1991), CD3+T cells predominated in third trimester decidua, with CD14+ macrophages and CD56+ uNK cells also present in substantial numbers. The presence of high numbers of CD56+ uNK cells in late pregnancy was striking as others have reported that CD56+ uNK cells decline after the first trimester and are virtually absent at term (Haller *et al.* 1993, Saito 2000). The present findings are, however, in agreement with the view that uNK cells are present at term (Sindram-Trujillo *et al.* 2003) but that their numbers are maximal during the period of trophoblast invasion and spiral artery remodelling that is characteristic of the first 20 weeks of pregnancy.

The observation of reduced uNK cell numbers in PE and FGR concurs with a recent report of reduced uNK cells in pregnancies complicated by FGR with or without PE (Eide *et al.* 2006); it should be noted that the PE study group in this study had growth-restricted babies. However, the findings contrast with those of Stallmach *et al.* (1999) and Bachmayer *et al.* (2006) who reported increased numbers of CD56+ cells in PE decidua compared with controls. In the absence of information on the FGR status of the PE study groups in these latter studies, use of placental bed biopsy and the avidin–biotin method, which is more sensitive than the PAP method used by Stallmach *et al.* (1999), may also partly account for the differences in uNK cell numbers. Reduction in CD56+ leucocyte numbers in PE with

Table 1 Comparison of CD3/CD56, CD8/CD56 and CD161/CD3 double labelling between pre-eclampsia (PE) and normal third trimester decidua.

mAb	Control third trimester	Pre-eclampsia	P value
CD3/CD56	1.27 $\pm$ 0.27 ^a	1.50 $\pm$ 0.26	0.55
CD8/CD56	2.56 $\pm$ 0.90	0.75 $\pm$ 0.25	0.04
CD161/CD3	5.70 $\pm$ 0.52	4.91 $\pm$ 0.65	0.36

^aNumbers represent mean number of positive cells per $\times 250$ field $\pm$ S.E.M.

FGR and FGR uncomplicated by PE may be important in the pathogenesis of these conditions; failure of spiral artery 'transformation' occurs in the first half of pregnancy, long before clinical presentation (Brosens & Renner 1972). In murine pregnancy, spiral artery remodelling appears to be initiated by an uNK cell-dependent mechanism that is mediated, at least in part, by IFNG (Ashkar *et al.* 2000). Furthermore, IFNG induces nitric oxide synthase (NOS) activity, leading to the production of NO (Nunokawa & Tanaka 1992). As well as being a powerful vasodilator, NO inhibits proliferation of rat vascular smooth muscle cells and chronic inhibition of NOS induces hypertension and FGR in animal models (Yallampalli & Garfield 1993). A reduction in uNK cell numbers may also contribute to deficient spiral artery remodelling due to reduced production of angiogenic growth factors including vascular endothelial growth factor (VEGFA), angiopoietin-2 (ANGPT2) and placenta growth factor (PGF; Sharkey *et al.* 1993, Li *et al.* 2001, Lash *et al.* 2006a). Thus, reduction in the number of uNK cells could set up a cascade of events leading to altered trophoblast invasion and vascular remodelling (Hanna *et al.* 2006), ultimately resulting or contributing to vasoconstriction and PE or FGR in later pregnancy.

The reduction in CD14+ cell numbers in PE is consistent with a flow cytometric study (Burk *et al.* 2001) but conflicts with other immunohistochemical reports (Reister *et al.* 1999, Wilczynski *et al.* 2003, Lockwood *et al.* 2006, Kim *et al.* 2007). In common with Burk *et al.* (2001), we identified placental bed macrophages by CD14 immunopositivity specific for the endotoxin receptor expressed by macrophages, whereas others (Reister *et al.* 1999, Lockwood *et al.* 2006) used an antibody against CD68, which reacts with lysosome-associated proteins and is a marker of phagocytic cells (Saito *et al.* 1991). Reister *et al.* (1999) also focused on macrophage numbers around myometrial spiral arteries while in the present study leucocyte populations were quantified in decidua basalis. Lockwood *et al.* (2006) described numerous focally clustered CD68+ cells in superficial decidua attached to the delivered placenta and noted that their distribution in controls varied widely, ranging from absent to sparse to numerous. The discrepancy in findings between studies may also partly reflect the difference sources of decidual tissue. Recently, Kim *et al.* (2007) have reported the presence of CD14-/CD68+ and CD14+/CD68+ cells in the placental bed of women with PE and preterm labour. Interestingly, CD14-/CD68+ cells predominated in the decidua while the reverse was found in the superficial myometrium. CD14 negative macrophages, as well as having reduced phagocytic activity, do not produce key cytokines known to regulate trophoblast invasion e.g. transforming growth factor- β (TGFB) and TNF (Smythies *et al.* 2005). As with uNK cells, the reduced number of decidual macrophages may

contribute to a local reduction in NO in PE (Haddad *et al.* 1995).

In addition to vascular remodelling, decidual leucocytes may also regulate EVT invasion. In human pregnancy, uNK cells produce significant amounts of IFNG (Saito *et al.* 1993) and the receptor for IFNG is present on EVT (Hampson *et al.* 1993) suggesting that IFNG could be involved in invasion. Reports have both supported and refuted a direct role for IFNG in regulation of EVT behaviour. We have recently demonstrated that IFNG regulates EVT invasion via mechanisms dependent on increased EVT apoptosis as well as decreased protease activity (Lash *et al.* 2006b). By contrast, a report examining EVT column formation and migration found IFNG to only partially regulate EVT (Hu *et al.* 2006). TNFA-related apoptosis inducing ligand (TNFSF10), expressed by decidual macrophages and T cells, has been shown to have stimulatory effects on trophoblast, increasing trophoblast invasion and migration and also inducing activation of insulin-like growth factor II and matrix metalloproteinase I genes, which are also involved in successful placentation (Phillips *et al.* 1999). Reduced numbers of decidual macrophages and T cells in PE may thus lead to decreased levels of TNFSF10 expression thereby leading to a reduction in trophoblast invasion and perturbed placental function.

Representing the smallest immune cell population in first trimester human decidua, T lymphocytes have been the subject of little research. One of the major functions of CD3+ and CD8+ T-lymphocytes in decidua is likely to be cytokine production. The reduced levels of T cells concur with the recent report of decreased numbers of FOXP3+CD3+ regulatory T cells in decidua in PE (Sasaki *et al.* 2007). Others have reported that decidual lymphocytes of women with PE produced increased levels of IFNG with reduced levels of IL6 and IL10 indicating cytokine balance dysregulation (Wilczynski *et al.* 2003).

Double labelling of CD3+CD56+, CD8+CD56+ and CD161+CD3+ leucocytes revealed a small population of these double-positive cells in late pregnancy and PE decidua. Lack of TCR V α 24 positivity (data not shown) indicates that these double-positive cells probably do not represent an NKT population but rather an activated T-cell population (Pittet *et al.* 2000). There are no reports to date on the presence or absence of decidual NKT cells in PE, but only <0.5% of decidual lymphocytes in normal pregnancy express TCR V α 24 (Tsuda *et al.* 2001). The failure to detect decidual NKT cells in normal pregnancy may reflect the relative insensitivity of immunohistochemistry compared with flow cytometry as well as their low frequency. The small population of CD8+CD56+ cells was reduced in PE. Recently, distinct CD8+ T cell populations have been shown to display differential cytolytic ability (Pittet *et al.* 2000, Ohkawa *et al.* 2001) with CD8+CD56+ T cells

Table 2 Clinical details of subject groups.

	Normal third trimester	PE ^a	FGR
Number of subjects	12	12	8
Gestational age (weeks) (range)	37.0 (±1.0) (26–38)	34.2 (±1.3)* (27–41)	34.1 (±2.5) (31–38)
Birth weight (kg)	3.14 (±0.23)	2.23 (±0.33)*	1.34 (±0.38) [†]
Systolic blood pressure (mmHg)	115.8 (±3.1)	156.7 (±3.4) [†]	120.6 (±7.7)
Diastolic blood pressure (mmHg)	69.2 (±2.1)	103.7 (±2.0) [†]	70.6 (±7.7)
Proteinuria (g/24 h) ^b		2.5 (±0.7)	
Urate (mmol/l)		388 (±23)	
Platelets (×10 ⁹ /l)		152 (±19)	

* $P < 0.05$ compared with control third trimester pregnancy group. [†] $P < 0.001$ compared with control third trimester pregnancy group.

^aEight percent (one subject) also had FGR. ^b $n = 10$. PE defined by International Society for Study of Hypertension in Pregnancy (ISSHP) criteria. Data expressed as mean and $\pm$ s.d.

showing increased cytolytic activity. The significance of their reduced presence in PE is unclear.

Although small populations of B lymphocytes have been reported in decidua (Hussein *et al.* 2009), this study focused on the major cytotoxic lymphocyte populations, and due to the relative scarcity of decidual B lymphocytes we did not include these in our study.

The difference in gestational age between PE and control group is a potential limitation of this study. However, due to the earlier gestational age of delivery, before 34 weeks for women with early onset PE, this was unavoidable. The groups were matched for gestational age as closely as possible and it is in our opinion that the large difference in leucocyte numbers between PE and late pregnancy decidua represents a true finding, and is not due to the earlier age of PE samples.

In summary, the present findings indicate that both PE and FGR are associated with a reduction in specific decidual leucocyte subpopulations, particularly CD56+ uNK cells. These changes, through alterations in local cytokine and vasoactive mediators, may be implicated in the failure of spiral artery transformation and consequent vasoconstriction, which are characteristic of severe PE and FGR.

Materials and Methods

Subjects and sample collection

Placental bed biopsies were performed on 12 healthy women with no hypertension or FGR undergoing elective Caesarean section due to breech presentation or previous Caesarean section, 8 women with FGR without maternal hypertension and

12 women with PE undergoing Caesarean section in the third trimester of pregnancy, as described previously (Robson *et al.* 2002). None of the subjects was in labour. Placental bed biopsies contained interstitial trophoblast and at least one spiral artery. PE was defined as pregnancy-induced hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg after gestational week 20) and proteinuria (≥ 300 mg/24 h) in women with no other underlying medical disease (Brown *et al.* 2001). FGR was defined ultrasonically as foetal abdominal circumference (AC) < 10 th centile with a decrease in AC SDS of > 1.5 SDS and umbilical artery pulsatility index > 95 th centile (Robson & Chang 1995). One subject (8%) in the PE group also had FGR. Gestational age was matched as closely as possible between subject groups, but the gestational age for the control group was higher than that for PE and FGR groups. Gestational age was significantly lower in the PE group than the control group ($P < 0.05$).

Clinical details of the study groups are shown in Table 2. The study was approved by the Newcastle and North Tyneside Joint Ethics Committee and written consent was obtained from all women.

Tissue samples (5–10 mm³) were snap-frozen in liquid nitrogen-cooled isopentane and stored at -70°C . Cryostat serial sections (7 μm) were air-dried overnight, fixed for 10 min in acetone at room temperature, air-dried overnight and stored wrapped at -20°C .

mAb

Five murine mAbs were employed for immunostaining of frozen sections (Table 3). The optimal dilution for each mAb was determined in positive control tissue (frozen sections of

Table 3 Primary mAbs.

mAb	Specificity	Clone	Supplier	Dilution
CD3	T cells	UCHT1	Novocastra ^a	1:200
CD8	Cytotoxic T cells, small population of NK cells	DK25	Dako ^b	1:100
CD14	Macrophages, monocytes	UCHM1	Serotec ^c	1:20
CD56(NCAM1)	NK cells (including uNK cells), small population of cytotoxic T cells	ERIC1	Novocastra	1:100
CD161(KLRB1C)	C-type lectin expressed by NK, NKT and T cells. CD161+ cells represent activated T cells	B199.2	Serotec	1:100

^aNovocastra Laboratories Ltd, Newcastle-upon-Tyne, UK. ^bDako Ltd, Ely, UK. ^cSerotec Ltd, Kidlington, Oxford, UK.

either tonsil or normal early pregnancy decidua), and was selected on the basis of maximal specific reactivity and minimal background staining.

Single immunohistochemical labelling

Single immunohistochemical labelling was performed using an avidin–biotin complex (ABC) method (Vectastain Elite, Vector Laboratories, Peterborough, UK). Sections were rehydrated in 0.05 M Tris-buffered 0.15 M saline (pH 7.6 (TBS)) for 5 min, followed by incubation for 10 min with the supplied normal horse blocking serum to block non-specific binding sites. Slides were then incubated sequentially with appropriately diluted primary mAb (30 min), biotinylated horse anti-mouse immunoglobulins (30 min) and the Vectastain ABC-peroxidase reagent (30 min). The reaction was developed using 3,3'-diaminobenzidine (Sigma Chemical Co.) to give a brown reaction product. Sections were counterstained with Mayer's haematoxylin, blued in Scott's tap water, dehydrated, cleared and mounted in DPX synthetic resin (BDH, Poole, UK). Positive and negative (test sections in which the primary mAb was replaced by normal serum) controls were included in each immunostaining run and for each mAb.

Double immunohistochemical labelling

Due to the co-expression of CD8 and CD56 by both cytotoxic T-lymphocytes and NK cells (Pittet *et al.* 2000), and the identification of CD3+CD56+CD161+ NKT cells in human decidua (Tsuda *et al.* 2001), double immunohistochemical labelling was undertaken to characterise further the decidual leucocyte populations in control third trimester and PE samples. Sections were first labelled for CD3, CD8 or CD161 (30 min, except CD161, which was incubated overnight in a humid chamber at 4 °C) using the ABC-peroxidase method described above. The reaction was developed using NovaRed (Vector Laboratories) to give a red reaction product. The slides were then washed in TBS for 5 min, overlain with normal rabbit serum diluted 1:10 and incubated for 30 min with the second primary antibody, CD56, CD3 or CD8. Sections were then sequentially incubated with rabbit-anti-mouse immunoglobulins (Dako, Ely, UK; 30 min) and alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako; 30 min). The reaction was developed with alkaline phosphatase substrate III kit (Vecta Blue; (Vector Laboratories) to give a blue reaction product and the reaction was terminated by washing in water. Slides were mounted in Supermount (BioGenex, San Ramon, CA, USA), air-dried overnight and then permanently mounted with DPX.

Quantification and analysis of results

Positive cells were counted in five randomly selected medium power ($\times 250$) fields using a 10×10 mm graticule. Counting was performed in equivalent fields in serial sections from each case for each mAb; fields containing large glands and vessels were excluded. The mean (S.E.M.) number of positive labelled cells per $\times 250$ field was calculated for each cell population.

When counting double-labelled slides, both single-positive (red for first antibody, blue for second antibody) and double-positive cells (both blue and red) were counted. Data on cell counts were normally distributed and results were compared using Student's *t*-test. Differences were considered to be statistically significant at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Action Medical Research and an Edmund Harker Foundation studentship.

Acknowledgements

We are grateful to patients and staff for their assistance in collecting samples.

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Received 6 January 2009

First decision 9 February 2009

Revised manuscript received 25 March 2009

Accepted 8 April 2009