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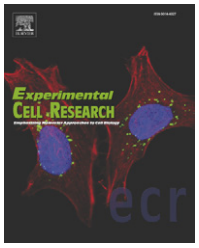

Research Article

A role for the endothelial glycosaminoglycan hyaluronan in neutrophil recruitment by endothelial cells cultured for prolonged periods

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ABSTRACT

Glycosaminoglycans (GAGs) presented on the surface of endothelial cells (ECs) are believed to influence leukocyte recruitment during inflammation, but their roles remain uncertain. Here we report an in vitro model of prolonged culture of human EC in which the contributions of heparan sulphate (HS) and hyaluronan (HA) to the process of neutrophil recruitment could be studied. Previously, we reported that increasing EC culture duration (up to 20 days) enhanced neutrophil recruitment in response to low dose (1 U/ml) but not high dose (100 U/ml) of tumour necrosis factor-α (TNF). Here we found that HS and HA were present at much higher levels on the surface of day 20 cultures than day 3 cultures. Neutrophil recruitment on both day 3 and day 20 ECs was mediated through CXCR chemokine receptors and interleukin-8 (IL-8). In addition, mRNA levels for TNF receptors, signalling pathway constituents, adhesion receptors, and chemokines involved in neutrophil recruitment were similar for day 3 and day 20 ECs. To test whether the enhanced neutrophil recruitment on day 20 EC was mediated by GAGs, they were removed enzymatically. Removal of HA (but not HS) inhibited neutrophil recruitment, as did antibody blockade of CD44, a counter-receptor for HA on neutrophils. Supernatants from hyaluronidase-treated day 20 ECs were more potent in activating neutrophils than supernatants from untreated EC. Thus, HA has a role in neutrophil recruitment that is revealed in long-term cultures where it increases potency of response to sub-optimal levels of TNF. This effect appears to occur through a dual mechanism involving chemokine presentation and interaction with CD44.

Introduction

Vascular endothelial cells (ECs) play a major role in the regulation of recruitment of leukocytes in inflammation. In response to cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), they upregulate expression of selectin receptors which support capture and rolling of flowing leukocytes (reviewed by [1]). They also synthesize and present activatory chemokines and other chemotactic agents that induce stable adhesion between leukocyte integrins and endothelial inter-cellular adhesion molecule 1 (ICAM-1) and promote subsequent migration (reviewed by [2,3]). There is increasing evidence that the glycosaminoglycans (GAGs) presented in the glycocalyx of vascular endothelium play a role in interactions with leukocytes [4,5] as well as in vascular permeability [6] and thromboresistance [7].

GAGs are strongly negatively charged, linear sugar chains composed of repeated sulphated and/or carboxylated disaccharide units. There are seven forms: hyaluronan (HA), chondroitin 4-

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sulphate, chondroitin 6-sulphate, dermatan sulphate, keratan sulphate, heparin, and heparan sulphate (HS) [8]. With the exception of HA, glycosaminoglycans are covalently attached to a protein backbone to form proteoglycans [9,10]. There are a number of studies which suggest a role for GAGs in leukocyte recruitment. GAGs expressed on ECs have been shown to be able to bind chemokines which may have a role in leukocyte activation, including RANTES (CCL5), MCP-1 (CCL2), IL-8 (CXCL8), and MIP-1α (CCL3) [11-13]. In mice producing a mutant form of HS with a reduced ability to bind chemokines, neutrophil infiltration into tissue in response to a number of inflammatory stimuli was impaired [14]. In vitro, heparinase treatment of TNF-stimulated glomerular ECs caused a reduction in the number of rolling and firmly adherent leukocytes [15,16]. On the other hand, an earlier study in mice showed that degradation of endothelial HS by injection of heparitinase actually increased the number of adherent leukocytes directly observed in venules [17]. Subsequent infusion of heparan sulphate or heparin tended to reverse this effect, and it was suggested that the intact glycocalyx might act as an adhesive shield. The heparan sulphate proteoglycan syndecan-1 has been shown to regulate leukocyte binding in inflammation in a number of studies, possibly through its presentation of HS and modulation of integrin binding to ICAM-1 (e.g., [18,19]). In mice lacking syndecan-1, there was increased leukocyte recruitment in models of myocardial infarction [18] and of oxazolone-mediated delayed-type hypersensitivity [19].

HA may also play a role in leukocyte recruitment. Administration of exogenous HA reduced neutrophil adhesion in the post-capillary venules of the hamster cheek pouch or adhesion to TNF-stimulated HUVEC [20]. Treatment of mice with hyaluronidase reduced neutrophil recruitment in the cremasteric microcirculation, and a similar result was observed in mice lacking expression of CD44 [21]. CD44 is the best characterised ligand of HA and is found on leukocytes as well as ECs [4]. The interaction between CD44 on neutrophils and HA highly expressed on the surface of ECs in liver sinusoids was shown to play a major role in neutrophil sequestration in the liver of mice [22].

The foregoing indicates that while GAGs have the potential to modulate leukocyte recruitment via several routes (e.g., immobilising chemokines, binding leukocyte counter-receptors or forming a physical hindrance in the glycocalyx), the importance of each may be hard to separate, and some literature reports appear contradictory. To study the role(s) of GAGs we took advantage of a previously described adhesion model incorporating prolonged cultures of human umbilical vein endothelial cells (HUVECs) [23]. HUVECs have been shown to produce and present HS [12,24], and presentation was increased in our long-term cultures [23]. Functionally, we found that during long-term culture, adhesion and migration of neutrophils on HUVEC that had been treated with low dose of TNF became more efficient, although this potentiation was not evident with high doses of TNF. The adhesion receptors used did not change during prolonged culture, but activation of neutrophils became more effective [23]. Here, comparing 3-day and 20-day cultures, we found that HA on the endothelial surface increased as well as HS, and it was the former rather than the latter which mediated the increase in efficiency of recruitment of neutrophils. The activatory chemokines utilised were similar, but HA appeared able to present stimuli to neutrophils and also to provide a ligand for CD44.

Materials and methods

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [25] and maintained in Medium 199 (M199; Invitrogen, Paisley, UK) containing 20% fetal calf serum, 28 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 1 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone (all from Sigma-Aldrich, Poole, UK) until confluent. Primary cultures were dissociated with trypsin/EDTA (Sigma) and passaged into tissue culture multi-well plates (Falcon; Becton Dickinson Labware, NJ, USA) as described [23]. Seeding density yielded confluent monolayers within 24 hours. ECs were cultured in M199 as above for either 3 or 20 days and then treated with TNF (0, 1, or 100 U/ml, equivalent to 0, 50, or 5000 pg/ml; Sigma) for 4 hours prior to assay. Each experiment used first-passage ECs from a different donor.

Neutrophil isolation

Blood was collected from healthy volunteers into K_2EDTA (Sarstedt, Leicester, UK) and used within 2 hours. Neutrophils were isolated by centrifuging the whole blood over a 2-step density gradient consisting of equal quantities of Histopaques 1119 and 1077 (Sigma) as described [26]. The neutrophil layer was aspirated from the density interface and washed twice in PBS containing 1 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 0.15% culture-tested bovine serum albumin (Sigma; PBS/BSA). Neutrophils were counted using a Coulter counter (Coulter Electronics, Harpenden, UK) and adjusted to 10^6/ml in PBS/BSA.

Adhesion and migration of neutrophils

ECs cultured in 6-well plates were washed with PBS/BSA to remove TNF and placed on the stage of a phase-contrast videomicroscope enclosed at 37 °C. An adhesion assay was performed as previously described [23]. Briefly, neutrophils (2 ml) were added (time = zero) and allowed to settle for 5 minutes. The monolayer surface was washed twice by gentle swirling, aspiration and replacement of PBS/BSA, to remove non-adherent cells. A series of 5 video fields were recorded at time = 25 minutes, when transendothelial migration is complete in this system [23]. The large-well format and brief settling period allowed efficient washing of the central, recorded regions of the plates, and produced very low levels of non-specific binding to unstimulated EC but allowed high levels of adhesion to TNF-treated EC (see Results).

The videomicroscopic recordings were analysed offline using a computer-assisted image analysis system (ImagePro; DataCell, Finchampstead, UK). Neutrophils in each field were counted, and the average was taken for the repeated fields. The average was converted to counts per squared millimeter using the known field dimensions, multiplied by the area of the well, divided by the total number of neutrophils added, and then multiplied by 100 to express neutrophil adhesion as a percentage of the cells added. The adherent neutrophils were classified into two groups (i) phase bright cells on the surface of the endothelium and (ii) transmigrated phase dark cells that were highly spread and migrating under the endothelial monolayer [27]. Efficiency of migration was defined as the percentage of adherent cells

that had transmigrated, and thus represented a measure of migration that was independent of the level of adhesion *per se*.

Antibody treatments

Neutrophils were treated with an Ab against CD44 (clone IM7.8.1; gift from Aideen Long, Trinity College Dublin, The University of Dublin) or control rat IgG (Chemicon International, CA, USA), CXCR1 or CXCR2 (2 μg/ml both Biosource International, CA, USA), or an isotype-matched antibody against α_v-integrin (10 μg/ml; L230; Chemicon) for 20 minutes at room temperature. Alternatively neutralising monoclonal antibody (mAb) against IL-8, epithelial neutrophil-activating peptide 78 (ENA-78), growth-related oncogene-α (GRO-α) (all 10 μg/ml; R&D Systems, Abingdon, UK), or an isotype-matched antibody against VCAM-1 (Dako UK Ltd., Ely, UK) was added to ECs during the last 15 minutes of TNF treatment.

Evaluation of gene expression by reverse transcriptase–polymerase chain reaction (RT–PCR)

RNA was extracted from EC using TRIzol (Invitrogen), and reverse transcription of single-stranded cDNA and PCR were conducted as described [28]. Primers and PCR conditions for β-actin, E-selectin, ICAM-1, CD31, IL-8, ENA-78, GRO-α, and P-selectin were as previously published [29].

Quantification of EC glycosaminoglycans

Quantification of EC GAGs was performed on live ECs grown in 96-well plates (Falcon; Becton Dickinson). Biotin-conjugated HA binding protein (at a concentration of 1:50; Sigma) or mAbs against HS (0.5 μg/ml; Europa Bioproducts Ltd, Ely, Cambridge) were added to the EC monolayer for 1 hour, on ice. Cells were washed with PBS/BSA and HRP-conjugated streptavidin or secondary horseradish peroxidase (HRP)-conjugated, goat–anti-mouse antibody (1:2000; Dako) was added for 1 hour, on ice, and washed off with PBSA. Peroxidase substrate (1,2-phenylenediamine dihydrochloride; Dako) was added for 7 minutes and the reaction was stopped by the addition of 100 μl of 1 M H_2SO_4. ELISAs were carried out in batches, with comparisons between culture durations and treatments made on the same plate, to reduce assay variation between experiments. Absorbance of the coloured product was measured at 490 nm using a plate reader.

Enzymatic digestion of GAGs on EC

Following 4 hours of TNF treatment, the TNF-containing medium was removed from ECs and the monolayer was rinsed with PBS/BSA. The monolayer was then treated with 2 ml of PBS/BSA with or without 30 mU/ml heparinase (Oxford Glycosciences, Oxford, UK) or 30 U/ml hyaluronidase (Sigma) for 15 minutes immediately prior to the assay. Supernatants were collected for use in subsequent analysis.

Neutrophil activation assay

Supernatants from hyaluronidase-treated or control EC monolayers were added to isolated neutrophils (2×10^6/ml) (1:1 ratio). After 5 minutes, neutrophil morphology was assessed using phase-contrast microscopy. Cells were classified as 'resting' if smoothly spherical or 'activated' if they had undergone shape change and had irregular outline [30].

Statistical analysis

When cultures from a particular day (day 3 or day 20) were exposed to a single treatment, e.g., enzymatic digestion, then results for treated cells and untreated controls were compared by paired *t* test. When day 3 and day 20 cells were compared to each other, e.g., for level of adhesion, an unpaired Student's *t* test was used, as the day 3 and day 20 cells were HUVECs from different donors and not necessarily tested on the same occasion with the same donor of neutrophils. Effects of multiple treatments were tested by analysis of variance (ANOVA), and when indicated, post-hoc comparison to control was done by Dunnett's test.

The statistical analysis was carried out using Minitab software (Minitab Ltd., Coventry, UK).

Results

Effect of EC culture duration and the role of chemokines in neutrophil recruitment

ECs maintained in culture for 20 days showed little change in morphology or cell number, when compared to those cultured for a shorter duration, but effects on neutrophil recruitment in response to TNF were apparent, as previously reported [23].

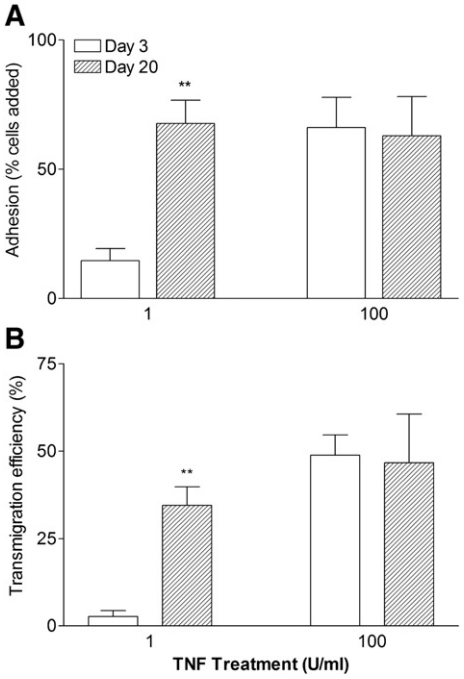

Fig. 1 – Effect of duration of EC culture on adhesion and migration of neutrophils on ECs treated with TNF. EC were cultured for 3 or 20 days then treated with 1 or 100 U/ml TNF for 4 hours before analysis of (A) neutrophil adhesion (expressed as a percentage of added cells) or (B) efficiency of neutrophil transmigration (expressed as a percentage of adherent cells). Data are mean ± SEM from 3 or 4 experiments. **$p<0.01$, compared to day 3 by Student's *t* test.

Following stimulation with 1 or 100 U/ml TNF for 4 hours, there were dose-dependent changes in adhesion and migration that were markedly influenced by the prior duration of culture. Specifically, there was an increase in adhesion and transmigration in response to 1 U/ml TNF on ECs cultured for 20 days, compared to 3 days. The response to 100 U/ml was the same regardless of culture duration (Figs. 1A and B). Very few neutrophils adhered to unstimulated ECs, regardless of culture duration (adhesion <1% for at least 3 experiments on each of day 3 and day 20 cultures).

We previously showed that adhesion receptors used for attachment were similar for ECs cultured for 3 or 20 days, but that increased recruitment of neutrophils at 20 days at 1 U/ml TNF was attributable to increased activation and stabilisation of adhesion [23]. We thus examined the chemokines and their receptors operating at 1 U/ml TNF. Blockade of neutrophil chemokine receptors CXCR1 and CXCR2 inhibited neutrophil adhesion (Fig. 2A) and transmigration (Fig. 2B) on both 3-day and 20-day cultures. Inhibition of transmigration was greater with CXCR2 blockade, compared to CXCR1 (Fig. 2B). Neutralising-antibody against IL-8 caused significant reductions in adhesion and transmigration on both day 3 and day 20 cultures (Figs. 3A and B). Neutralising GRO-α had no effect on adhesion or transmigration on either day 3 or day 20 cultures. Neutralising ENA-78 had more variable effects: there was a significant effect on adhesion on day 3 and not day 20, and a significant effect on transmigration on day 20 and not day 3. However, combined neutralisation of ENA-78 and IL-8 essentially abolished adhesion and migration for either duration of culture (Figs. 3A and B). Thus, although there may have been some variation between effects of individual receptors or chemokines between culture durations, the mechanisms of activation were similar. We conclude that from the most reliable data, e.g., with combined antibodies, joint actions of CXCR1 and CXCR2, and of IL-8 and ENA-78, were operative and underlie activation for both culture durations.

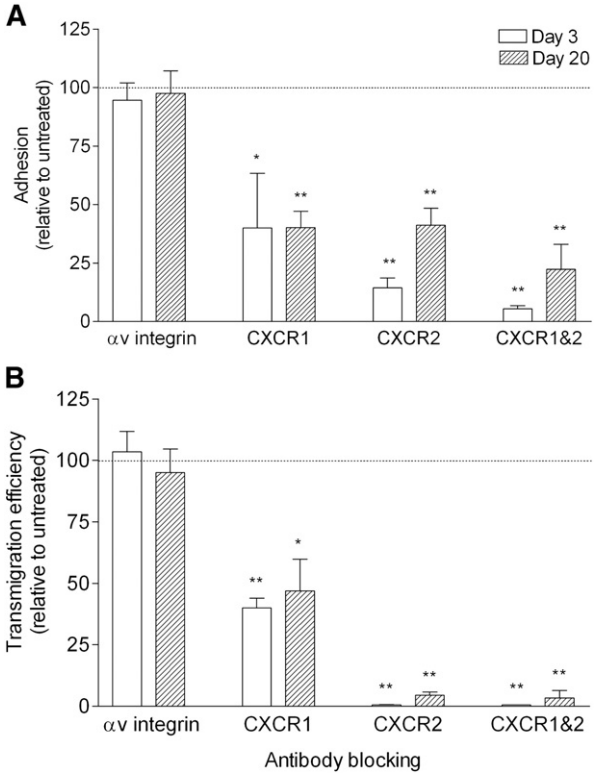

Fig. 2 – Effects of blocking antibodies against CXCR1 and/or CXCR2 on (A) adhesion or (B) transmigration of neutrophils on ECs cultured for 3 or 20 days, prior to stimulation with 1 U/ml TNF. Neutrophils were treated with mAb against CXCR1, CXCR2, or an isotype-matched antibody against α_v-integrin for 15 minutes prior to assay. Data are mean ± SEM for 3 experiments expressed relative to untreated cells. ANOVA showed a significant effect of treatment ($p<0.01$) but not of culture duration. *$p<0.05$, **$p<0.01$, compared to control by Dunnett's test.

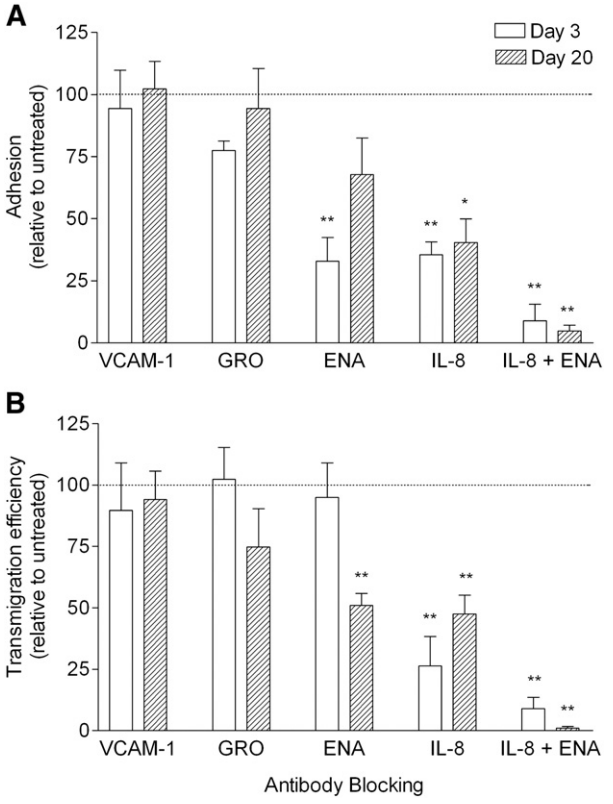

Fig. 3 – Effects of neutralising antibodies against IL-8, ENA-78, and GRO-α on (A) adhesion or (B) transmigration of neutrophils on ECs cultured for 3 or 20 days, prior to stimulation with 1 U/ml TNF. ECs were treated with mAb against IL-8, ENA-78, or GRO-α or an isotype-matched antibody against VCAM-1 for 15 minutes prior to assay. Data are mean ± SEM for 3 experiments expressed relative to untreated cells. ANOVA showed a significant effect of treatment ($p<0.01$) but not of culture duration. *$p<0.05$, **$p<0.01$, compared to control by Dunnett's test.

Effect of culture duration on expression of 'inflammatory' genes

Next, we analysed levels of EC mRNA to assess whether there were changes which would suggest an amplified responsiveness to 1 U/ml TNF in ECs that had been cultured for long periods. In four experiments, we found no consistent changes in the expression of TNF receptors (TNFR1 and TNFR2), or transcription factors of the NFκB/Rel family, or of the NFκB regulatory protein IκBα between day

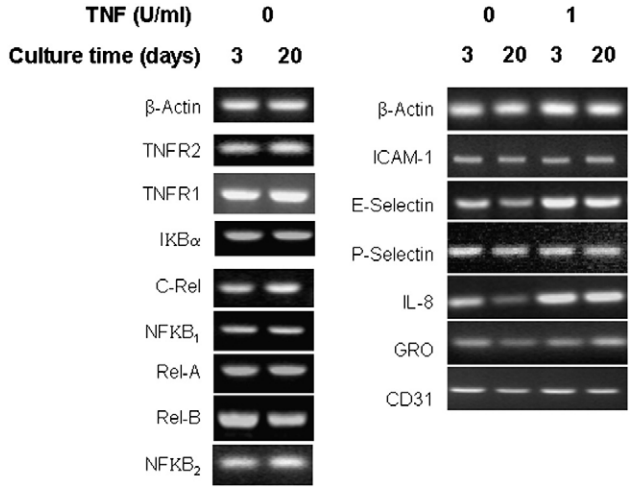

Fig. 4 – Expression of regulators or markers of inflammation in ECs cultured for 3 or 20 days. mRNA was isolated from resting ECs and analysed for TNF receptors 1 and 2, IκBα and the NFκB/Rel family of transcription factors, or from resting or TNF-treated ECs and analysed for ICAM-1, E-selectin, P-selectin, IL-8, GRO-α, and CD31. β-Actin is shown as a loading control. Expression of mRNA for ENA-78 was not detectable by this method. Gels are representative of 4 experiments.

3 and day 20 for unstimulated cultures. Typical results are illustrated in Fig. 4A. Although there were occasional variations between 3-day and 20-day cultures, densitometric analysis of gel bands confirmed that there were no significant effects of culture duration overall (data not shown). This suggests that no change occurred in the activation pathway for TNF. Moreover levels of expression of EC adhesion molecules or chemokine were similar for day 3 and day 20 ECs treated with 0 or 1 U/ml TNF (Fig. 4B). As expected TNF treatment caused up-regulation of mRNA, e.g., for E-selectin and IL-8, but basal and stimulated levels were similar for day 3 and day 20 cultures.

Role of glycosaminoglycans in changes in endothelial function

Having found no evident differences in mechanisms underlying neutrophil adhesion and transmigration linked to culture duration, or changes in gene expression that could explain increased recruitment at low dose TNF, we investigated whether changes in GAGs might have contributed. ECs which had been cultured for 3 or 20 days were assayed for surface expression of hyaluronan (HA) or heparan sulphate (HS). On day 3 cultures, HA was just detectable (Fig. 5A), but HS was undetectable (Fig. 5B). In limited experiments, increasing development time of the ELISA increased all signals but did not bring the signal for HS above the non-specific control. Both HA and HS showed much increased expression by day 20. The surface levels could be reduced to those at day 3 using the enzymes hyaluronidase and heparinase, respectively (Fig. 5). Expression levels of HA were not affected by the 4 hours of TNF treatment used for adhesion assays (data not shown).

In order to establish whether HS or HA on day 20 cultures could have a role in the increased recruitment of neutrophils, in response to low doses of TNF, we treated day 20 ECs with heparinase or hyaluronidase prior to adhesion assay. Heparinase treatment had no effect on neutrophil adhesion or transmigration on day 20 ECs

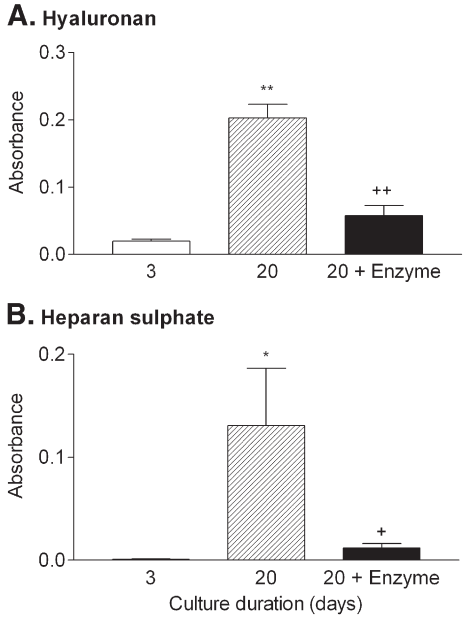

Fig. 5 – Effect of culture duration on endothelial surface concentrations of (A) hyaluronan or (B) heparan sulphate. ECs were cultured for 3 or 20 days before quantification, using biotin-conjugated HA binding protein or mAb against HS. The effect of pre-treatment with hyaluronidase or heparinase was also tested. Data are mean ± SEM for 3 to 6 measurements. **$p < 0.05$, compared to day 3 by Student's *t* test. $^{+}p < 0.05$, $^{++}p < 0.01$, compared to day 20 by paired *t* test.

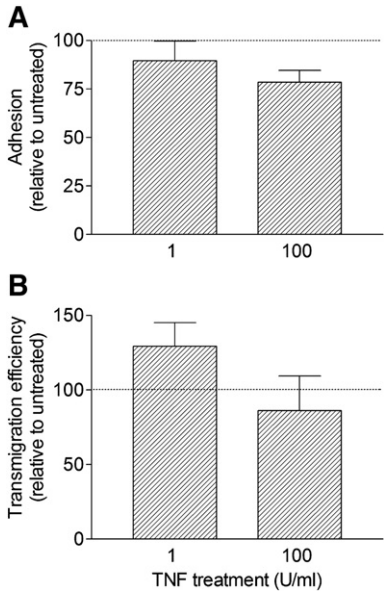

Fig. 6 – Effect of heparinase treatment of ECs on (A) adhesion or (B) transmigration of neutrophils. ECs were cultured for 20 days, stimulated with 1 or 100 U/ml TNF for 4 hours, then treated with PBS/BSA with or without 30 mU/ml heparinase for 30 minutes prior to assay. Adhesion and transmigration efficiency are shown for heparinase-treated ECs relative to untreated ECs. Data are mean ± SEM from 3 experiments.

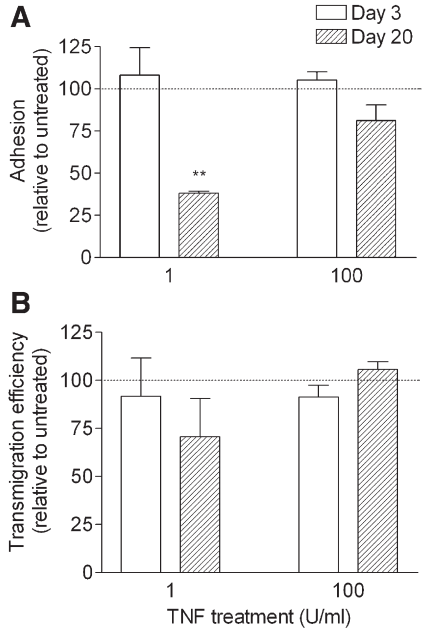

Fig. 7 – Effect of hyaluronidase treatment of ECs on (A) adhesion or (B) transmigration of neutrophils. ECs were cultured for 3 or 20 days, stimulated with 1 or 100 U/ml TNF for 4 hours, then treated with PBS/BSA with or without 30 mU/ml hyaluronidase for 30 minutes prior to the assay. Adhesion and transmigration efficiency are shown for heparinase-treated ECs relative to untreated ECs. Data are mean ± SEM from 4 experiments. **p < 0.01, compared to untreated by paired *t* test.

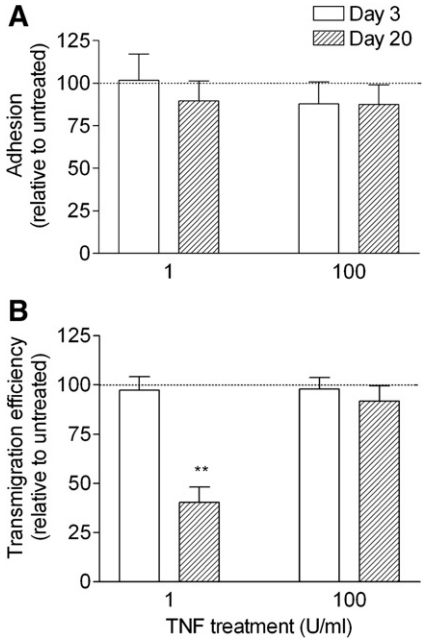

Fig. 8 – Effect of antibody against CD44 on (A) adhesion or (B) transmigration of neutrophils. ECs were cultured for 3 or 20 days and stimulated with 1 or 100 U/ml TNF. Neutrophils were treated with antibody against CD44 for 30 minutes prior to assay. Adhesion and transmigration efficiency are shown for antibody-treated neutrophils relative to untreated cells. Data are mean ± SEM from 3 or 4 experiments. **p < 0.01, compared to untreated by paired *t* test.

treated with 1 or 100 U/ml TNF (Fig. 6). However, following treatment with hyaluronidase, adhesion to day 20 ECs stimulated with 1 U/ml TNF was reduced to less than 50% of the untreated (Fig. 7A). There was no effect of hyaluronidase treatment on neutrophil recruitment to ECs stimulated with 100 U/ml TNF or to day 3 cultures treated with either dose of TNF (Figs. 7A and B).

To test whether the HA ligand on neutrophils, CD44, played a role in their recruitment on 20-day ECs, neutrophils were treated with an antibody against CD44. There was no effect of antibody treatment on the level of neutrophil adhesion to day 3 or day 20 ECs stimulated with 1 or 100 U/ml TNF (Fig. 8A). However, antibody specifically reduced transmigration efficiency through day 20 ECs treated with 1 U/ml TNF but not for 100 U/ml TNF or day 3 EC (Fig. 8B). Isotype-matched control antibody had no significant effect on either parameter (data not shown).

Neutrophil activation by supernatants from EC

We wondered whether HA might act by presenting chemokines to neutrophils. We took the supernatants from hyaluronidase-treated or untreated ECs and measured their ability to activate freshly isolated neutrophils. ECs stimulated with TNF released soluble mediators in the 30 minutes after removal of the TNF which were able to activate neutrophils (Fig. 9). Activation was more potent for EC treated with 100 U/ml TNF than 1 U/ml, but in the absence of hyaluronidase treatment, the supernatants had equal activity for 3-day or 20-day cultures. However, the supernatants from day 20 cultures treated with hyaluronidase induced higher levels of neutrophil activation than the untreated supernatants (Fig. 9). Hyaluronidase treatment did not increase the potency of supernatants from day 3 cultures. Nor did hyaluronidase itself increase neutrophil activation (Fig. 9). Importantly, supernatants from the

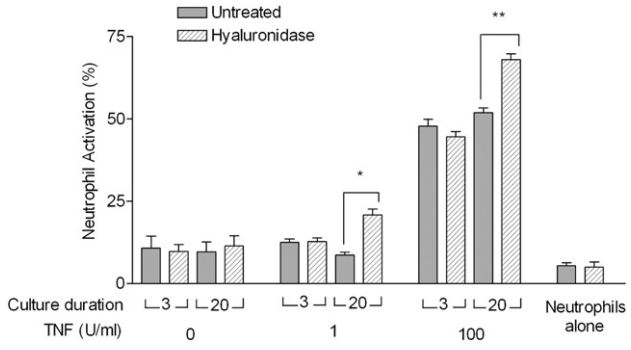

Fig. 9 – Effects of hyaluronidase on the ability of supernatants from ECs to activate neutrophils morphologically. ECs were cultured for 3 or 20 days and treated with 1 or 100 U/ml TNF for 4 hours. Medium was removed and replaced with PBS/BSA with or without 30 mU/ml hyaluronidase for 30 minutes. The supernatants were collected and added to neutrophils for 5 minutes and their shape change was observed using a microscope to determine the percent activated. For comparison, neutrophils were also treated with PBS/BSA with or without hyaluronidase. Data are mean ± SEM for 3 experiments. *p < 0.05, **p < 0.01, for hyaluronidase-treated vs. untreated by paired *t* test.

digestion of HA on unstimulated EC did not activate neutrophils, showing that there was no intrinsic action of HA fragments (Fig. 9). Thus, a neutrophil-activating ability was associated with HA for TNF-treated EC which had been cultured for 20 days.

Discussion

We have described an experimental model in which prolonged culture of endothelial cells increases their presentation of GAGs. This allowed us to study the contributions of heparan sulphate and hyaluronan to the regulation of leukocyte adhesion and migration. HA and HS were barely detectable on early cultures of HUVECs but well expressed after 20 days of culture. On 20-day cultures, HA promoted neutrophil recruitment induced by a low dose of TNF, but at a higher dose, the presence of HA was not required for an effective response. HA appeared to make the presentation of activator(s) to neutrophils more efficient, which was more critical at the lower level of stimulation. Put differently, the presence of HA explained the increase in efficiency of recruitment found in 20-day compared to 3-day cultures responding to TNF [23]. On the other hand, HS did not have an obvious role in neutrophil adhesion or migration in this model, for short- or long-term cultures.

Expression of HS on HUVECs has been reported previously [12,24]. Others did not detect HA on the surface of HUVECs after short-term culture, but it was detected on microvascular ECs, where expression increased after activation with agents including TNF [31]. Here, HA expressed on our 20-day cultures was removed by treatment with hyaluronidase but not increased by TNF treatment. There may be intrinsic differences between specific EC types, in terms of expression of HA and its counter-receptor CD44. Previous studies indicated that the ability of CD44 to bind HA was controlled in a cell-type and activation-specific manner, so that not all cells which expressed CD44 could bind HA [32]. This may explain why HA is particularly important for neutrophil recruitment in some tissues, such as the liver sinusoids, where it is constitutively present at high levels [22].

We previously reported that as endothelial culture duration was prolonged, efficiency of neutrophil recruitment at a low dose of TNF was progressively increased [23]. Under flow conditions, capture of neutrophils was not affected by culture duration (i.e., presence or absence of GAGs on the EC surface), but neutrophil activation and transmigration was more efficient for prolonged cultures [23]. Here we found that the chemokine receptors CXCR1 and CXCR2 were equally important in neutrophil recruitment on 3- or 20-day cultures, as were the chemokines IL-8 and ENA-78. Upon studying EC mRNA for TNF signalling pathway constituents, adhesion receptors, and chemokines, we found no difference between day 3 and day 20 cultures. Therefore, we hypothesized that it was the increased levels of GAGs that led to the enhanced activation of neutrophils. Treatment of the day 20 ECs with hyaluronidase reduced neutrophil adhesion in response to low levels of TNF, whereas treatment with heparinase had no effect. Hyaluronidase treatment of day 20 TNF-stimulated ECs also yielded supernatants that were more potent neutrophil activators than untreated supernatants. This enzyme-potentiated activity was not evident for day 3 cultures or for unstimulated day 20 cultures. The latter ruled out intrinsic activating effects of HA fragments. Thus, it seems that HA may bind and improve efficiency of presentation of chemokines to neutrophils. Such a concept is consistent with in vivo studies where intravenous injection of HA inhibited firm adhesion (but not rolling) of neutrophils in post-capillary venules of the hamster cheek pouch [20], perhaps by competing with HA on the endothelial surface.

In our studies, the effect of HA digestion was only seen at the lower concentration of TNF, suggesting that HA was not necessary for efficient neutrophil recruitment at the higher concentration. In response to high levels of stimulation, such as 100 U/ml TNF, the ECs produce very large quantities of chemokines that presumably do not need to be presented by GAGs to have a physiological effect on neutrophils. Indeed, day 3 cultures were capable of recruiting neutrophils at a high level of stimulation, despite having a low concentration of GAGs on their surface. The day 3 cultures did not efficiently recruit neutrophils after a lower level of stimulation, perhaps because they did not efficiently present chemokines via GAGs. In vivo, one would expect ECs to have an intact glycocalyx and to be exposed to levels of stimulation closer to 1 U/ml TNF. As a guide, TNF can be detected in the serum of patients with tumours at levels up to about 50 pg/ml (equivalent to 1 U/ml for the TNF used here) [33–35] or 250 pg/ml immediately after injection of *Escherichia coli* endotoxin [36]. The long-term cultures used here generated a basement membrane that is lacking in short-term cultures [23] and were quiescent in the sense that they did not recruit flowing leukocytes unless treated with TNF. Thus, we propose that the 20-day 'endothelium' and the lower dose of TNF are particularly relevant to inflammation in vivo and that our results suggest a hitherto unexpected role for HA under such conditions.

Treatment of neutrophils with antibody against CD44, the major ligand for HA, reduced neutrophil recruitment under the same circumstances as treatment of EC with hyaluronidase; for day 20 but not day 3 ECs, and only for a low dose of TNF. However, the antibody reduced transmigration rather than adhesion itself. Thus, while its effect relied on the presence of HA, the loss of CD44–HA interaction inhibited the later stage of migration but did not disrupt neutrophil activation so as to reduce stable attachment itself. On the other hand, completely removing HA caused this pro-adhesive signal to be lost. Neither type of interaction was necessary at high levels of TNF. Evidence for distinct actions of endothelial HA and neutrophil CD44 is available from studies of neutrophil recruitment in venules of mice lacking CD44 (either totally, on their EC or on the neutrophils) or treated with hyaluronidase [21]. Lack of CD44 on EC or treatment with hyaluronidase caused loss of stable adhesion in venules compared to untreated WT mice, while hyaluronidase treatment had no further effect on mice already lacking CD44. On the other hand, if only neutrophils lacked CD44 (achieved by transplantation of CD44-negative bone marrow into wild-type mice) adhesion was maintained but migration into tissue was reduced. Thus, endothelial HA increased efficiency of adhesion, while neutrophil CD44–HA interaction increased transmigration of adherent cells. While many cell lines that express CD44 can bind HA, resting leukocytes cannot [37,38]. For monocytes at least, activation with inflammatory cytokines such as TNF and IL-1 induced CD44–HA binding [39]. Resting murine neutrophils could not bind to HA [21], and thus, an initial interaction with chemokines may be required for CD44–HA binding.

Based on the above, we suggest that, in our model, neutrophils binding to ECs became activated and firmly adherent through the

action of surface-presented chemokines (which was promoted by HA), and this enabled a later stage of CD44–HA binding that facilitated transmigration. To seek direct evidence that HA on day 20 ECs did present chemokines, we assayed IL-8 in endothelial supernatants and whole cell lysates (with and without hyaluronidase digestion) after treatment with TNF. However, although we did tend to find less free IL-8 in day 20 than day 3 supernatants, we did not detect a compensatory greater level in whole cell lysates or in hyaluronidase digests from the surface (data not shown). In our experience, HUVECs produce and release plentiful IL-8, and it is likely that only a very small proportion is retained and functionally active at the cell surface in any case. Our sandwich ELISA assay may not have been sensitive enough to detect small changes in concentration. The direct detection of chemokine binding on the surface of EC is problematic and typically requires electron microscopy or radioimmunoassay (e.g., [40,41]). In the absence of definitive evidence, it is also worth considering other mechanisms by which HA may have modulated neutrophil binding here. HS bound to syndecan-1 has been found to modulate leukocyte integrin binding to ICAM-1 [19]. It is possible that HS bound to CD44 could also affect stable adhesion in such a way, although in the case noted above, the effect of the proteoglycan was inhibitory. A positive signal might alternatively arise from signalling through HA binding to neutrophil CD44, which has been shown previously to increase cytokine production and cell motility [42,43]. The exact mode of action of HA and the possible effects of binding to CD44 on interaction with endothelium thus require further clarification.

To our knowledge, this is the first report of a role for HA in recruitment of human leukocytes to EC. It suggests two contributions: through improved activation of neutrophils and subsequent action as a receptor for CD44. The restricted circumstances under which effects were detectable (long-term culture and relatively low dose TNF) explain why such roles have not been detected before but are likely to be relevant to inflammatory responses in vivo, as suggested by analogous intravital observations in mice [21]. Although we also found HS detectable in greater quantities on long-term cultures, there was no effect of heparinase treatment on neutrophil adhesion or migration. Others found inhibition of neutrophil adhesion to short-term cultures of glomerular EC after heparinase treatment [15,16]. Impairment of sulphation of HS in mice was associated with reduced recruitment of neutrophils into inflamed tissue [14], but in a separate study using direct observations of inflamed venules, neutrophil adhesion actually increased after enzymatic digestion of HS [17]. Thus, while there is mounting evidence that GAGs fulfill important roles in leukocyte recruitment in inflammation, contributions may be site- or stimulus-specific. It is also apparent that variations in culture conditions in vitro can alter the ability of ECs to recruit leukocytes. Previously we found that long-term culture also induced deposition of a distinct basement membrane that altered behaviour of migrated neutrophils [23]. Thus, recapitulating responses and mediators operating in vivo can be difficult experimentally, and care must be taken in generalising results obtained in vitro.

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