base-pair fragment corresponding to the mRNA encoding $VEGF_{165}$ (data not shown): $VEGF_{165}$ is a secreted form of the growth factor and stimulates proliferation of endothelial cells[14]. The potential targets of VEGF is the population of nearby cells expressing VEGF receptors. VEGF receptors are widely distributed on all vascular endothelial cells of adult rat tissues, including quiescent endothelium, but are not found on non-endothelial cells[14,15]. To visualize blood capillaries better we hybridized adjacent serial sections with a von Willebrand factor (VWF)-specific probe which exclusively identifies endothelial cells irrespective of their proliferation status. In Fig. 2, capillary bundles are seen to be preferentially clustered alongside the stripes of VEGF-expressing cells. Immunohistochemical labelling has shown that VEGF, which is produced elsewhere in the tumour, is concentrated in tumour blood vessels[12]. It is therefore tempting to speculate that the proximity of a high concentration of capillaries to sites of VEGF production is the result of a local angiogenic response elicited by the angiogenic factor.

The marked differences in steady-state levels of VEGF mRNA among otherwise indistinguishable tumour cells (Figs 1, 2) can be correlated with their proximity to necrotic centres, where oxygen supply is minimal. We have interpreted this observation to mean that VEGF could be specifically induced in response to hypoxia. It is equally possible, however, that VEGF is induced by factor(s) released from necrotic cells. To distinguish between these two possibilities, we determined whether VEGF expression is regulated by oxygen. Glioma cells were grown under normoxic and hypoxic conditions, and levels of VEGF mRNA were subsequently measured by RNA-blot analysis. Steady-state levels of VEGF mRNA were significantly increased within 18 h of growth under low oxygen tensions (Fig. 3). A 13-fold induction was measured under complete anoxia (as achieved by oxidation with a palladium catalyst). The increase in VEGF mRNA levels was reversible. Upon re-exposure of cells to normal oxygen tension, VEGF expression resumed its low constitutive level (Fig. 3). No evidence for cell death could be detected in cultures exposed to anoxia, and cells continued to proliferate at a normal rate following re-exposure to normal oxygen tension.

These findings support the thesis that the induction of VEGF in gm tumours occurs in response to hypoxia. They also indicate that the rate of release of angiogenic factors by tumour cells might in general be variable, being constantly adjusted according to the changes in the cell microenvironment.

To determine whether VEGF is hypoxia-inducible in other cell types, we did a similar experiment with a cell line of skeletal muscle myoblasts (L8). As shown in Fig. 3, VEGF was also hypoxia-inducible in these cells (5-fold induction after standardization for RNA loads). Similar levels of induction were also observed in a fibroblast (mouse L-cells) line and in primary cultures of cells derived from rat heart muscle (data not shown). We conclude that VEGF is hypoxia-inducible in a variety of cell types. These results suggest that, in principle, VEGF might play a part in ischaemia-induced collateralization responses taking place in normal muscle and connective tissues.

It has been proposed that basic fibroblast growth factor (bFGF) is released by tumour cells, ischaemic myocardium and other tissues in which ischaemia has caused extensive cell damage[16]. We could not detect any significant increase in steady-state levels of bFGF mRNA at sites where expression of VEGF was upregulated (data not shown). This finding does not exclude, however, the possibility that cell damage causes the release of pre-made bFGF from its stores[17].

The mechanism(s) that control responsiveness of VEGF to hypoxia are unknown. Precedents for other hypoxia-inducible genes include examples of both transcriptional activation (such as platelet-derived growth factor B-chain gene[18]) and post-transcriptional regulation by a *trans*-acting protein that physically interacts with the gene (erythropoietin for example[19]). Preliminary results with a general inhibitor of protein synthesis have indicated that VEGF induction depends on prior protein synthesis (Fig. 3). It also remains to be determined whether increased mRNA production is due to low oxygen, or is secondary to increased lactate in the environment (as in the case of angiogenesis mediated by macrophages recruited to hypoxic wounds[20]) or to accumulation of other metabolites.

Finally, VEGF has potent vascular pemeabilization activity[21,22], and hypoxaemia is associated with increased vascular permeability[23,24]. It is possible that VEGF functions as the link connecting hypoxia and increased vascular permeability. The apparent leakiness of blood vessels in gm tumours[4] could also be accounted for by the large amounts of VEGF secreted by the tumour cells. □

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Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*

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CLINICAL and experimental studies suggest that angiogenesis is a prerequisite for solid tumour growth[1,2]. Several growth factors with mitogenic or chemotactic activity for endothelial cells *in vitro* have been described, but it is not known whether these mediate tumour vascularization *in vivo*[3,4]. Glioblastoma, the most common and most malignant brain tumour in humans, is distinguished from astrocytoma by the presence of necroses and vascular proliferations[5,6]. Here we show that expression of an endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), is

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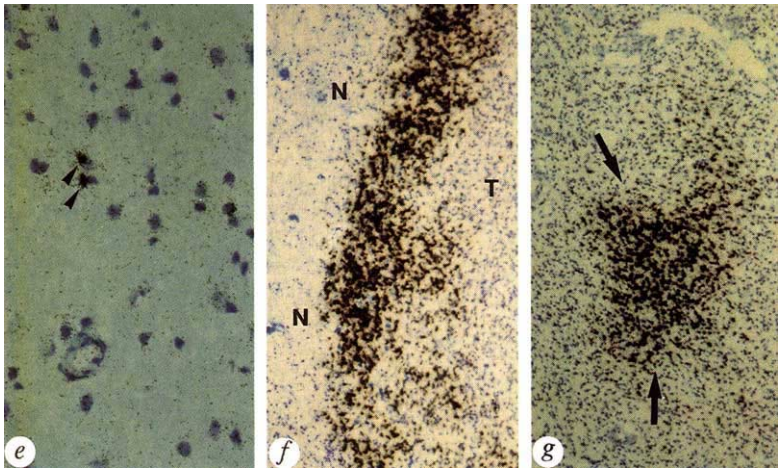


FIG. 1 VEGF mRNA is expressed in some astrocytoma cells but is significantly upregulated in a subset of glioblastoma cells *in vivo*. Frozen sections from low-grade and high-grade glioma hybridized *in situ* with VEGF cRNA. *a–d* (facing page), Low-power microphotographs from low-grade glioma (astrocytoma WHO grade II; *a–b*) and high-grade glioma (glioblastoma multiforme WHO grade IV; *c, d*) show that only a few astrocytoma cells, which cannot be seen at the magnification in *a*, express VEGF mRNA. In contrast, in glioblastoma, VEGF expression is dramatically upregulated in tumour foci (arrowheads in *c*). The tumour cell focus marked with an arrow is shown at higher magnification in *g*. *a* and *c*, Hybridization with antisense RNA; *b* and *d*, control hybridization with the sense probe. *e–g* (left), High-power magnification of astrocytoma (*e*), and two different glioblastomas (*f,g*). In astrocytoma (*e*), VEGF mRNA is expressed in a small number of tumour cells ►

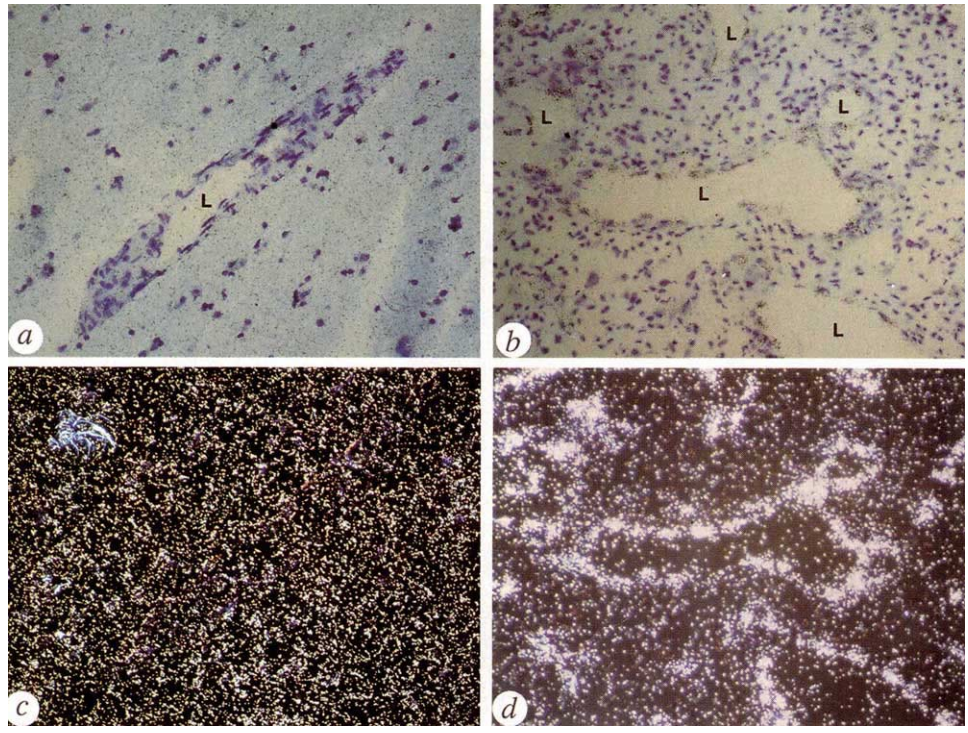


FIG. 2 VEGF-receptor mRNA is expressed in tumour endothelial cells but not in normal brain endothelial cells *in vivo*. *In situ* hybridization with a VEGF-receptor (*flt*) cRNA on normal brain and glioblastoma. VEGFR mRNA is not expressed in the cortex and white matter from normal adult brain (we did not systematically examine human brain and can therefore not comment on VEGF expression in other regions of the brain) (*a,c*). In glioblastoma, VEGFR mRNA expression is confined to tumour vascular endothelial cells; tumour cells show no hybridization (*b,d*). *a–b*, Bright-field and *c–d*, darkfield images, respectively; L, vessel lumen.

METHODS. Serial sections from the same specimens described in Fig. 1 legend were used for *in situ* hybridization. A 1,080-bp fragment of the human *flt* gene (corresponding to nucleotides 3,233–4,313; ref. 31) was amplified by polymerase chain reaction from human placenta cDNA, cloned into pBluescript vector (Stratagene) and used to generate antisense RNA probe. Exposure time was 10 days. No specific hybridization was observed with the control sense RNA probe.

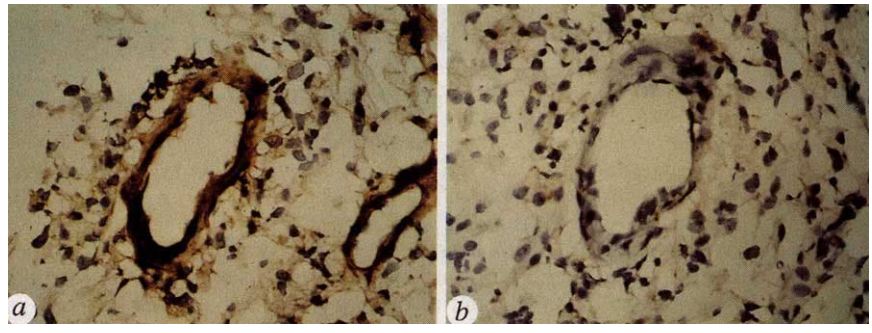
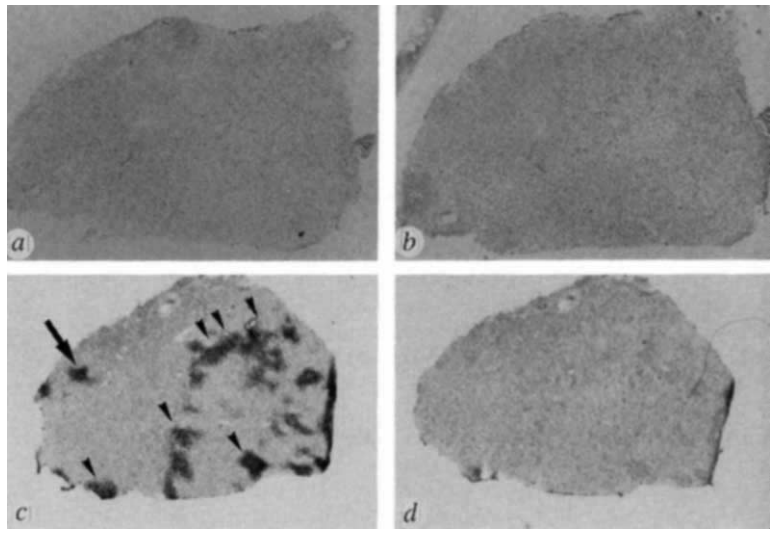


FIG. 3 VEGF protein is present on the tumour vasculature *in vivo*. *a*, Immunocytochemical staining for VEGF shows strong immunoreactivity in the vasculature of a glioblastoma. Glioma cells show only faint immunoreactivity, undistinguishable from background staining. *b*, The immunostaining could be totally abolished by preincubation of the antibody with the peptide. Immunostaining for VEGF is significantly lower in astrocytoma and undetectable in normal brain vasculature (not shown).

METHODS. Frozen sections were fixed in acetone for 7 min at −20 °C. An antipeptide antibody directed against the amino-terminal 20 amino acids of human VEGF (sequence APMAEGGGQNHHEVVKFMDV) was raised in rabbits and affinity-purified on nitrocellulose coated with 80 μg immobilized human recombinant $VEGF_{165}$. The purified antibody was applied on sections at a concentration of 5 μg ml^{-1}. Immunohistochemistry was carried out with a streptavidin–biotin method as described[12]. For control purposes, the antibody was incubated with 100 μg ml^{-1} peptide for 15 min at room temperature.

► (indicated by arrowheads). In glioblastomas, clusters of glioma cells with abundant VEGF mRNA expression were observed. VEGF upregulation was seen in palisading cells that reside along necroses (*f*) and in groups of glioblastoma cells that were not localized around obvious necroses (arrows in *g*). N, necrosis; T, tumour.

METHODS. Fresh tumour specimens (two astrocytomas, WHO grade II; two anaplastic astrocytomas, WHO grade III; and four glioblastomas, WHO grade IV) and one normal brain specimen (frontal cortex plus underlying white matter from a 33-year-old female who died of non-neurological disease, obtained 13 h post-mortem) were embedded in Tissue-Tek and frozen in liquid nitrogen. Frozen sections were hybridized *in situ* as described[15]. A cDNA clone encoding $VEGF_{165}$ (ref. 18) was used to generate an antisense cRNA probe. Exposure time was 14 days. Sense-RNA probe was used as a control and showed no specific hybridization.



induced in astrocytoma cells but is dramatically upregulated in two apparently different subsets of glioblastoma cells. The high-affinity tyrosine kinase receptor for VEGF, *flt*, although not expressed in normal brain endothelium, is upregulated in tumour endothelial cells *in vivo*. These observations strongly support the concept that tumour angiogenesis is regulated by paracrine mechanisms and identify VEGF as a potential tumour angiogenesis factor *in vivo*.

Angiogenesis, the sprouting of capillaries from pre-existing blood vessels, occurs during embryonic development and certain physiological conditions such as menstruation[4,7,8]. In addition, endothelial cells proliferate under pathological conditions such as wound healing, eye disease and arthropathy[7,8]. Angiogenesis is also a prerequisite of solid tumour growth[2,9]. It has been suggested that the onset of angiogenesis is an early event in tumorigenesis[9,10] and may facilitate tumour progression and metastasis[1,11]. It is unknown, however, how angiogenesis is regulated *in vivo*[7].

Tumour angiogenesis may be regulated by growth factors that are secreted by tumour cells[2,8]. Several endothelial growth factors with angiogenic activity have been described (reviewed in refs 3 and 4). These include fibroblast growth factors, platelet-derived growth factor (PDGF)[12,13] and vascular endothelial growth factor (VEGF). VEGF is a dimeric glycoprotein with structural homology to PDGF. Several variants of VEGF have been identified which arise by alternative splicing of messenger RNA[14]. We considered VEGF to be a candidate for a tumour angiogenesis factor *in vivo* because (1) the expression pattern of VEGF during mouse development is consistent with a role in embryonic angiogenesis[15]; (2) VEGF mRNA is found in some primary tumours[16]; (3) it is produced by tumour cell lines *in vitro*[17,18]; (4) it is a secreted mitogen[15,17,18]; and (5) its mitogenic activity is apparently restricted to endothelial cells[19,20].

In humans, gliomas account for over 60% of primary intracranial neoplasms[5]. Gliomas most commonly arise by malignant transformation of astrocytes and are classified according to histopathological criteria as astrocytoma (low-grade glioma) or glioblastoma (high-grade glioma). Glioblastoma is defined as an astrocytic tumour with (1) high cell density, (2) cellular polymorphism, (3) mitoses, (4) necroses with palisading cells, and (5) prominent vascularization with endothelial cell proliferation[5,21]. The distinction between low-grade and high-grade glioma has severe implications for the treatment and prognosis of patients. Patients with low-grade glioma have a mean survival time of several years, and are treated by tumour removal. High-grade glioma patients have a mean survival time of less than one year and are treated by tumour removal, post-operative radiation and, occasionally, additional chemotherapy. Glioblastoma may arise *de novo*, but also forms by malignant progression from astrocytoma. During this progression a remarkable alteration of the vascular phenotype occurs, consisting of prominent endothelial cell proliferation and an increased vessel density[5]. The presence of necroses and endothelial cell proliferation are the most important histopathological criteria for distinguishing high-grade from low-grade glioma[6,21]. Inhibition of angiogenesis may therefore be an approach to destroying tumours that are highly vascularized and not treatable by conventional methods[22,23]. Glioblastoma seems to be the prototype of a tumour suitable for anti-angiogenic therapy[24].

To investigate whether VEGF is involved in glioma angiogenesis, we hybridized normal brain and glioma tissue *in situ* with ^{35}S-labelled antisense RNA (Fig. 1). VEGF mRNA is not present in the white matter of normal human brain (where gliomas normally arise), but is expressed in cells scattered in the cortex. In low-grade gliomas, VEGF is expressed in few tumour cells. In glioblastoma, we observed a dramatic upregulation of VEGF mRNA expression in two apparently different subsets of tumour cells. As is shown in Fig. 1, VEGF mRNA is strongly expressed in small anaplastic glioma cells which line up in close proximity to necrotic areas. These cells are known as palisading cells[5]. A different pattern of VEGF expression was observed in a glioblastoma, where VEGF is expressed in clusters of tumour cells.

Recently a member of the PDGF receptor family, *flt*, was identified as a high-affinity receptor for VEGF[25]. By northern analysis, a 7.0-kilobase (kb) *flt* mRNA transcript was found in comparable amounts in astrocytoma and glioblastoma (H.A.W. *et al.*, unpublished results). We performed *in situ* hybridization to investigate the cellular localization of *flt*. As is shown in Fig. 2*b*, VEGF receptor (*flt*) mRNA is strongly expressed in vascular cells in gliomas and the expression appears to be confined to endothelial cells. The amount of VEGF receptor mRNA seems to be similar in tumour endothelial cells in astrocytoma and glioblastoma, but we could not detect VEGF receptor mRNA in endothelial cells in normal brain (Fig. 2*a*). This finding suggests that expression of the VEGF receptor is induced in endothelial cells during tumour development.

To investigate whether VEGF protein reaches its target cells, we incubated tissue sections with an affinity-purified anti-peptide antibody directed against human VEGF. Figure 3 shows the strong immunostaining of tumour vascular endothelial cells in a glioblastoma. This finding is consistent with studies by Dvorak *et al.*[26], who showed, using a similar antibody, that the protein is present in the vasculature of guinea-pig line-10 tumours. Surprisingly, we found no immunoreactivity in glioblastoma cells. Indeed, given the strong expression of VEGF in

a subset of cells, one would expect to be able to detect VEGF immunocytochemically in those cells. Reasons for the failure to detect VEGF in producer cells may include (1) rapid secretion and diffusion of VEGF from producer cells; (2) failure of the antibody or the detection method to respond to small amounts of VEGF; and (3) accumulation of VEGF in blood vessels by binding to its receptor or by binding to heparan sulphate[26].

Two VEGF mRNA transcripts of 3.9 kb and 4.3 kb were found by northern analysis in glioma tissue. Both transcripts were increased up to 50-fold in glioblastoma compared with astrocytoma (H.A.W. *et al.*, unpublished results). Our *in situ* hybridization data confirm these analyses and show in addition that VEGF mRNA is selectively upregulated in a subset of malignant glioma cells during the transition of astrocytoma to glioblastoma. In particular, we observed localized VEGF mRNA upregulation in (1) palisading cells adjacent to necrotic areas and (2) in clusters of tumour cells without obvious adjacent necrosis. This localized upregulation of VEGF expression may be explained in two different ways. First VEGF expression may be inducible by hypoxia. The rapid cell proliferation in the centre of a tumour can lead to increased interstitial pressure, which may lead to compression closure of capillaries and consecutive tissue necrosis[27]. On the way to necrosis the increasing hypoxia may upregulate VEGF. Alternatively, upregulation of VEGF could be attributed to factors released from necrotic tumour cells. We observed only moderate levels of VEGF mRNA in a glioblastoma with radiation-induced (non-hypoxic) necroses (data not shown). This observation is consistent with the hypothesis that VEGF expression is hypoxia-inducible. Secondly, VEGF-expressing tumour cells could have a growth advantage and proliferate more rapidly than cells that do not express VEGF. Although the nature of palisading cells is not understood, it has been suggested on the basis of histopathological observations that these cells represent a subset of dedifferentiated anaplastic glioblastoma cells[5]. This observation, and our finding of selective VEGF upregulation without adjacent necrosis, is consistent with the hypothesis that VEGF-expressing cells may have a growth advantage. The clusters of VEGF-producing cells could thus represent an early glioblastoma state, which may finally lead to VEGF-producing cells surrounding a necrosis. It has been shown that progression of astrocytoma to glioblastoma involves a clonal expansion of tumour cells carrying mutations in the p53 tumour-suppressor gene[28]. As p53 mutations can inhibit gene expression in HCT116 human colon carcinoma cells *in vitro*[29], it will be interesting to investigate whether mutations in the p53 gene are associated with upregulation of VEGF in the same glioblastoma cells.

Our findings show that the expression of VEGF, an endothelial cell-specific mitogen, is induced in a low proportion of cells in low-grade glioma but is dramatically upregulated in a subset of anaplastic glioma cells. The fact that palisading cells express large amounts of VEGF may explain why endothelial cell proliferation in glioblastoma is particularly apparent in the vicinity of necroses[5,30]. In addition, we found that the expression of the high-affinity receptor for VEGF, *flt*, is induced in tumour vascular endothelial cells. These findings strongly support the concept that tumour angiogenesis is regulated by a paracrine mechanism, namely the expression and secretion of VEGF by glioma cells and its action on tumour vascular endothelial cells that express the VEGF receptor. Our results identify VEGF as a potential tumour angiogenesis factor in human gliomas *in vivo*. Direct proof for a role of VEGF in tumour angiogenesis will require the inhibition of VEGF-receptor activation *in vivo*. □

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Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets

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THE glycoprotein P-selectin is a cell adhesion molecule of stimulated platelets and endothelial cells, which mediates the interaction of these cells with neutrophils and monocytes[1,2]. It is a membrane component of cell storage granules[3–6], and is a member of the selectin family which includes E-selectin and L-selectin[7,8]. P-selectin recognizes both lineage-specific carbohydrate ligands on monocytes and neutrophils, including the Lewis x antigen, sialic acid, and a protein component[9–12]. In inflammation and thrombosis, P-selectin may mediate the interaction of leukocytes with platelets bound in the region of tissue injury and with stimulated endothelium[1,2]. To evaluate the role of P-selectin in platelet–leukocyte adhesion *in vivo*, the accumulation of leukocytes within an experimental thrombus was explored in an arteriovenous shunt model in baboons[13]. A Dacron graft implanted within an arteriovenous shunt is thrombogenic, accumulating platelets and fibrin within its lumen. These bound platelets express P-selectin[14]. Here we show that antibody inhibition of leukocyte binding to P-selectin expressed on platelets immobilized on the graft blocks leukocyte accumulation and inhibits the deposition of fibrin within the thrombus. These results indicate that P-selectin is an important adhesion molecule on platelets, mediating platelet–leukocyte