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# VWF RELEASE AND PLATELET AGGREGATION IN HUMAN MELANOMA AFTER PERFUSION WITH TNF $\alpha$

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## SUMMARY

Twenty-nine stage IIIA/B melanoma patients treated by isolated limb perfusion (ILP) with a high dose of recombinant human tumour necrosis factor alpha (rHuTNF $\alpha$ ), interferon  $\gamma$  (IFN $\gamma$ ), and melphalan were histologically documented with emphasis on therapy-induced changes of the tumour vasculature. Sequential biopsies were taken at various intervals before and after the treatment to compare the morphological changes. In order to visualize microvascular changes, immunostaining was performed for von Willebrand factor (VWF), type IV collagen,  $\alpha$ -smooth muscle actin, endothelial antigen PAL-E, tissue factor, CD41 (thrombocyte marker), and fibrin. In biopsies prior to perfusion, necrosis, haemorrhage, and fibrin thrombi were not found. Within 3 h following triple combination therapy, a change in the distribution of VWF staining occurred, from a discrete endothelial pattern in the untreated lesions to a fuzzy perivascular and subepidermal pattern in the treated lesions. Within 24 h, this was accompanied by intravascular thrombocyte aggregation and erythrocytosis, in the absence of tissue factor and fibrin deposits. These findings indicate that the thrombocyte aggregation observed is not caused by local procoagulant activity, but is rather the result of the therapy-associated vascular damage or haemostasis. Although it is difficult to derive the dynamics of this process from static images, we assume that TNF $\alpha$  induced endothelial cell damage, leading to VWF release. Released VWF may play a role in the adhesion between thrombocytes and the damaged endothelium or the denuded subendothelium. As a consequence, the blood flow is impaired, leading to congestion and oedema, compatible with an early stage of haemorrhagic infarction.

KEY WORDS—isolated limb perfusion; melanoma; TNF $\alpha$ ; VWF; CD41 and thrombocytes

## INTRODUCTION

The aim of the present study was to investigate the hypothesis that the tumour microvasculature is a target for tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) triple combination therapy. Changes in the microvasculature of the perfused melanoma lesions have already been observed, especially the upregulation of cellular adhesion molecules.<sup>1</sup> Here we report on

the morphological and immunohistochemical analyses of the microvascular coagulative events.

TNF $\alpha$  is a cytokine with pleiotropic actions, with direct and indirect antitumour effects, and is an important mediator of septic shock.<sup>2</sup> Systemic administration of TNF $\alpha$  in cancer patients is associated with severe toxicity and negligible anti-tumour effects. Phase I–II studies indicate that the maximal tolerated dose (MTD) in humans is  $\pm 350 \mu\text{g}/\text{m}^2$  intravenously.<sup>3</sup> Based on data in murine tumour models, one might expect a dose 10 to 50 times higher to be necessary to achieve

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Table I—Specification of the biopsy samples

Cases	Melanoma lesions ( <i>n</i> =60)	Patients ( <i>n</i> =29)
Before IFN $\gamma$ s.c.	<i>n</i> =6	<i>n</i> =6
After IFN $\gamma$ s.c., but before ILP	<i>n</i> =9	<i>n</i> =9
After ILP with IFN $\gamma$ , TNF $\alpha$ and melphalan		
Early biopsies $\leq$ 1 week	<i>n</i> =32	<i>n</i> =20
Late biopsies <1 year	<i>n</i> =10	<i>n</i> =10
Control patients after ILP with melphalan alone	<i>n</i> =3	<i>n</i> =3

Table II—The antibodies used

Antibody	Specificity	Reference
PAL-E	endothelium	9,19
A-2547 (Sigma)	$\alpha$ -smooth muscle actin	20
MCA 468/S/010 (Serotec)	CD41	21
BBA7 (British Bio-Technology)	CD31 (PECAM)	22
Tissue factor*	tissue factor	23
NKI-BETAB	100 kD melanosome associated glycoprotein	24
KH 58.01POS (CLB)	raHu VWF	25
PCO 01403 (Cappel)	collagen type IV	26
PoAb raHu fibrin	fibrin	27

\*A gift from Dr T. Edgington, MD, The Scripps Research Institute, California, U.S.A.

antitumour effects.<sup>4</sup> These differences may only be overcome in the setting of isolated limb perfusions (ILPs).

Isolated limb perfusion for stage III melanoma patients permits regional cytostatic concentrations 15 to 20 times higher than those reached after systemic administration. The standard drug in this setting is melphalan (L-phenyl-alanine mustard). ILP with melphalan alone produces a 40–50 per cent complete response rate with a median duration of local tumour control for all patients treated of only 6 months. True hyperthermia (>41.5°C) may improve the response rates, but may also lead to severe regional toxicity. The modest efficacy of ILP with melphalan alone in stage III melanoma led to the addition of high doses of TNF $\alpha$ . The results of the initial observations in stage III melanoma have recently been published by Liénard and Lejeune.<sup>5</sup> Interferon $\gamma$  (IFN $\gamma$ ) is added in this schedule because of the synergistic antitumour activity of IFN $\gamma$  plus TNF $\alpha$ .<sup>6</sup>

In experimental settings it has been frequently reported<sup>7</sup> that local elicitation of endothelial cell procoagulant activity by TNF $\alpha$  leads to the forma-

tion of intravascular thrombi, causing haemostasis and ischaemia *in vivo*. Alternatively, activation of the coagulation cascade or thrombocyte aggregation may be a consequence of induced vascular damage or of impairment of the blood flow. In order to determine whether and to what extent these phenomena occur in melanoma patients treated by ILP, we examined tissue specimens, sequentially taken before and after cytokine administration, using histological and immunohistochemical methods.

## PATIENTS AND METHODS

### *Patient characteristics*

Melanoma patients with in-transit metastasis of the limbs (stage IIIA), or with additional regional lymph node metastasis (stage IIIB), with no sign of distant metastasis, no other malignancies, and no severe arterial insufficiency were admitted for ILP.<sup>8</sup> In the three participating institutions (Jules Bordet Institute, Dr Daniel den Hoed Cancer Centre, NKI Amsterdam), all patients clinically

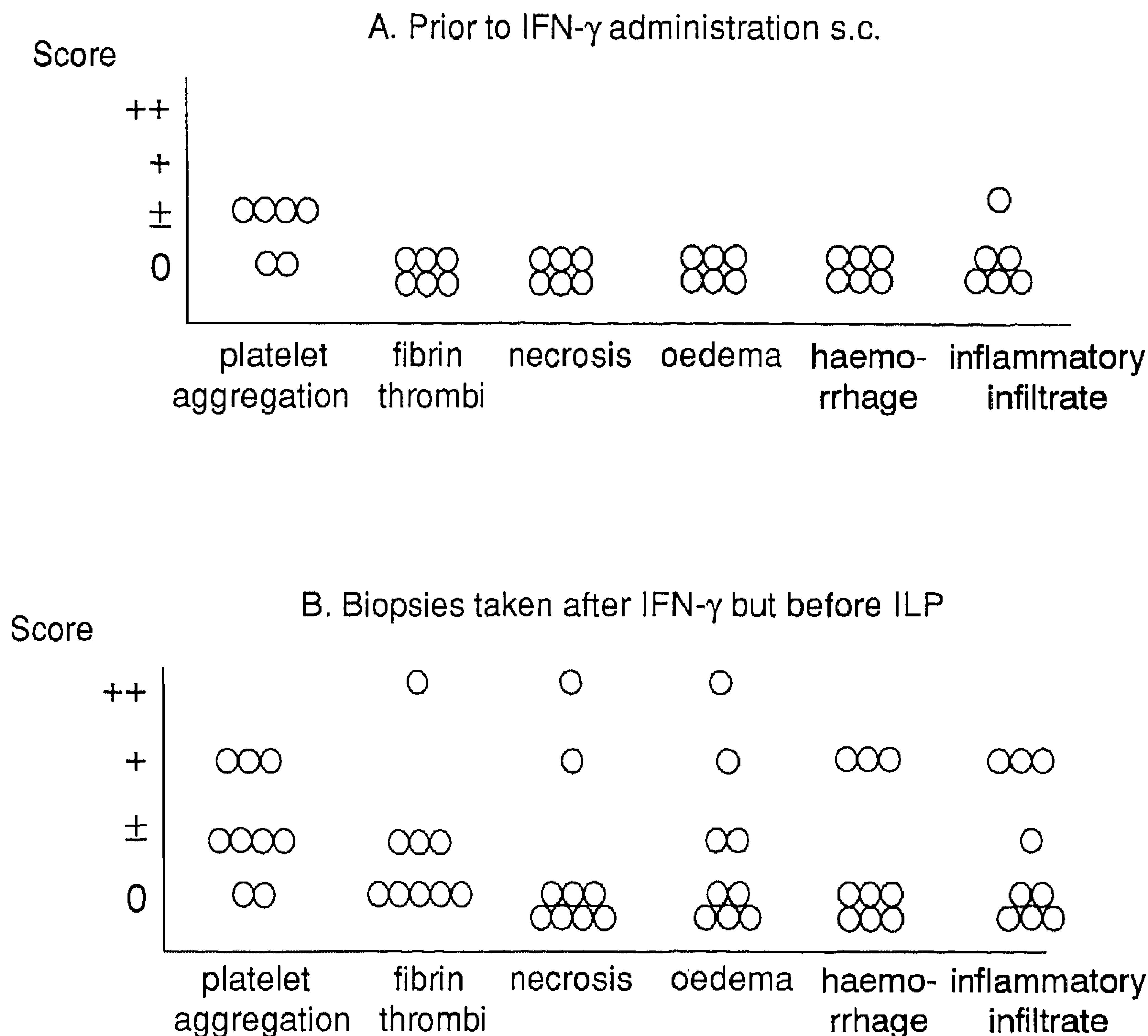


Fig. 1—Histopathological changes after various treatment regimes. (A) Prior to IFN $\gamma$  administration s.c.; (B) biopsies taken after IFN $\gamma$  but before ILP

selected for ILP were included in this morphological and immunohistochemical study. This study on the microvascular coagulative events comprises the biopsies of 29 melanoma patients. Other morphological phenomena have been described in 15 patients of this group by Renard *et al.*<sup>1</sup>

#### Drugs and treatment schedule<sup>1</sup>

**Drugs**—Recombinant human TNF $\alpha$  (0.2 mg/ampoule) and recombinant human IFN $\gamma$  (0.2 mg or  $1.5 \times 10^6$  U/ampoule) were a gift from Boehringer Ingelheim Germany. The cytostatic drug melphalan (Alkeran) was obtained as a sterile powder (100 mg) which was dissolved aseptically using solvent and diluent provided by Burroughs Wellcome (London, U.K.)

**Treatment schedule**—Patients received 0.2 mg IFN $\gamma$  subcutaneously (s.c.) on the 2 days prior to the ILP. ILP consisted of a 1.5 h long perfusion with 0.2 mg IFN $\gamma$ , 2 or 3 (arm)—4 mg (leg) TNF $\alpha$ , and 10 mg/l leg or 13 mg/l arm volume of melphalan at mild hyperthermia (40°C). IFN $\gamma$  and TNF $\alpha$  were injected successively as a bolus into the arterial line. Melphalan was administered 30 min later. At the end of ILP, the limb was washed thoroughly with Ringer's solution, Haemaccel, and/or 6 per cent dextran up to 4 l. Leakage of the drugs was measured with radioiodinated human serum albumin. Preoperatively and postoperatively dopamine was administered in about half of the patients at  $2 \mu\text{g}/\text{kg}$  per min by continuous infusion, usually for several hours up to 48 h. Fluid loading was applied before

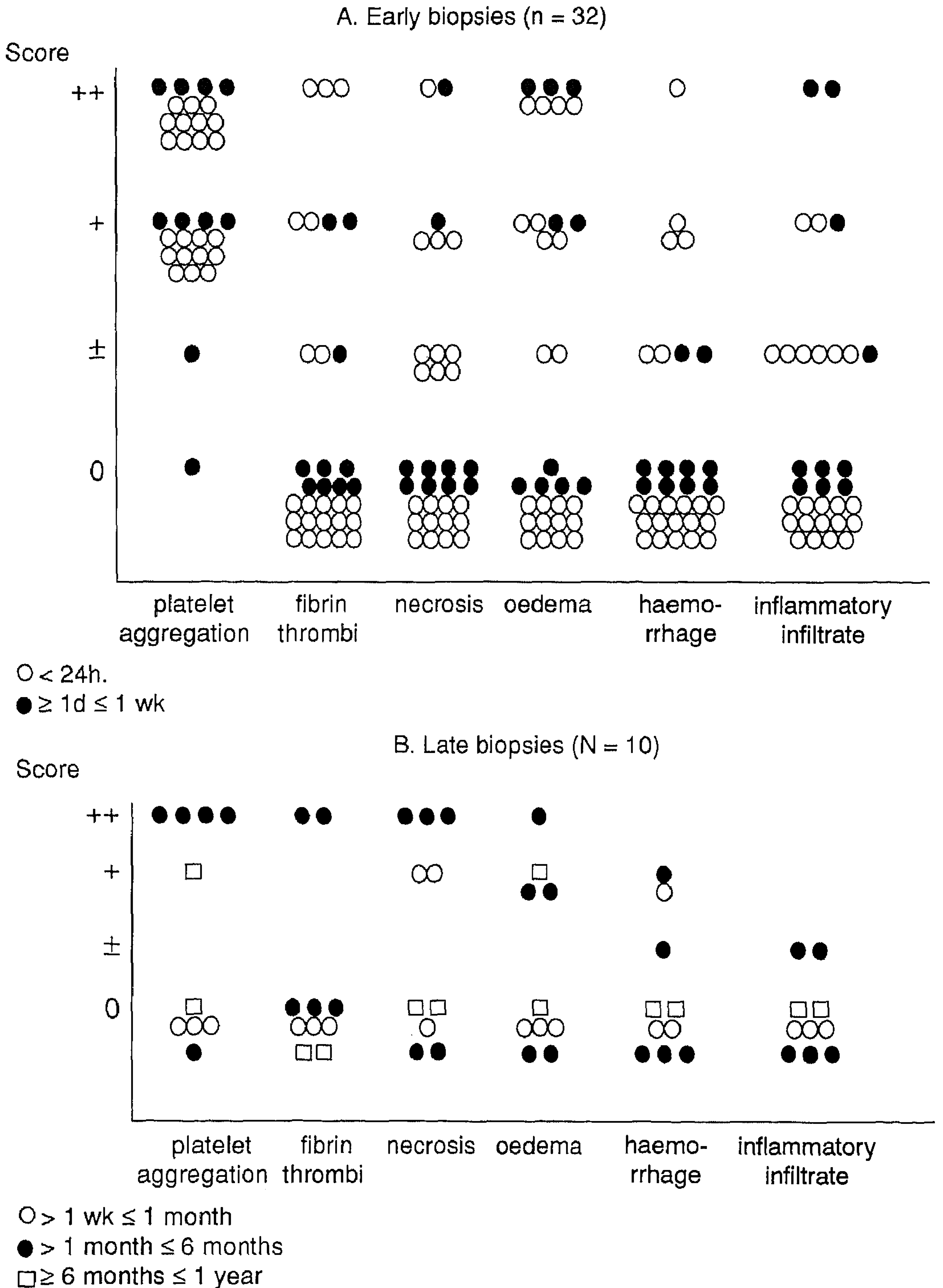


Fig. 2—Biopsies taken at various time points after perfusion with TNF $\alpha$ , IFN $\gamma$ , and melphalan. (A) Early biopsies; (B) late biopsies

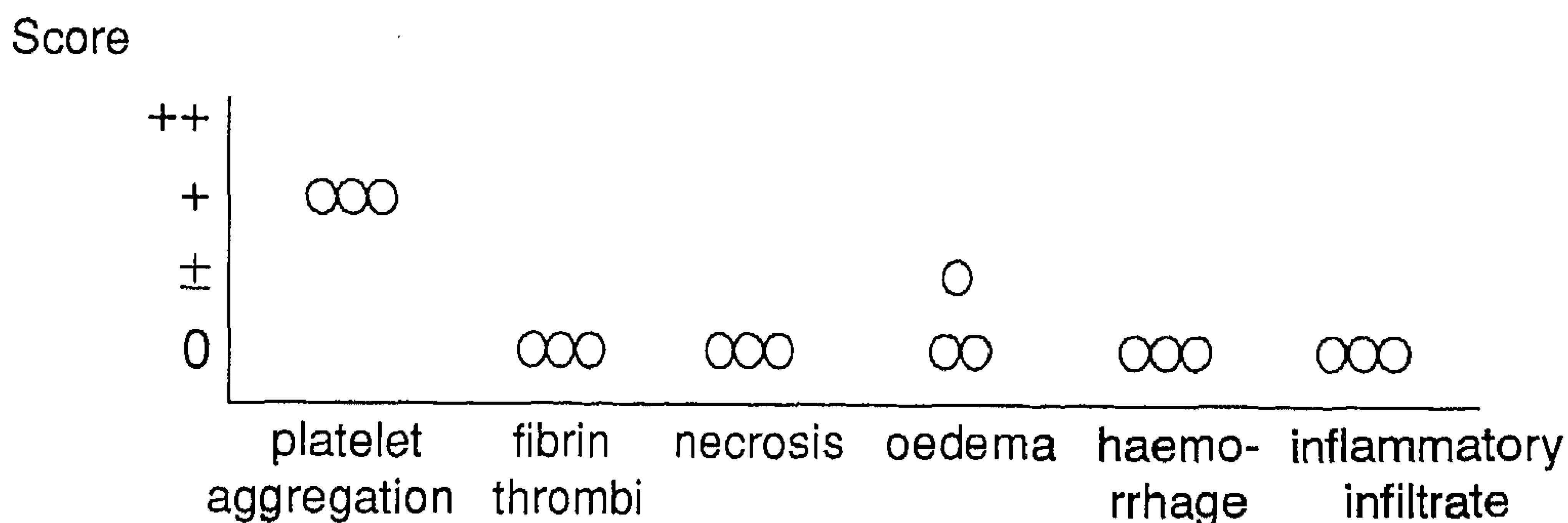


Fig. 3—Biopsies in patients after perfusion with melphalan alone

releasing the tourniquet after the completion of washing.

*Tissue specimens*—Oral informed consent was obtained from the patients to take sequential biopsies. Sixty biopsies from 29 patients were snap-frozen and stored at  $-80^{\circ}\text{C}$  until use. From 15 patients, besides frozen tissue, tissue samples were also taken to be embedded in paraffin after formalin fixation. The histological material consisted of biopsies taken before the administration of  $\text{IFN}\gamma$  s.c., after  $\text{IFN}\gamma$  s.c. but before ILP with  $\text{IFN}$ ,  $\text{TNF}\alpha$  and melphalan, and at various time points after perfusion (Table I).

#### Light microscopy

Consecutive  $4\mu\text{m}$  sections were cut and stained with haematoxylin and eosin. Besides documentation of the pathological diagnosis, the following histopathological features were assessed semi-quantitatively: necrosis (coagulative and/or haemorrhagic), thrombocyte aggregation (visualized by immunohistochemistry), fibrin thrombi, oedema, haemorrhage, and inflammatory infiltrate. The semi-quantitative grading used was as follows: 0 = no change;  $\pm$  = sporadic event; + = local event; ++ = generalized event.

#### Immunohistochemistry

The following antigens were studied in  $4\mu\text{m}$  frozen sections: endothelial antigen PAL-E, tissue factor, CD41 (thrombocytes), and  $\alpha$ -smooth muscle actin (pericytes and smooth muscle cells).

The following antigens were studied in  $4\mu\text{m}$  paraffin or frozen sections: von Willebrand factor, type IV collagen, fibrin, CD31 (PECAM), and 100 kD melanosome-associated glycoprotein.

The antibodies used are listed in Table II.

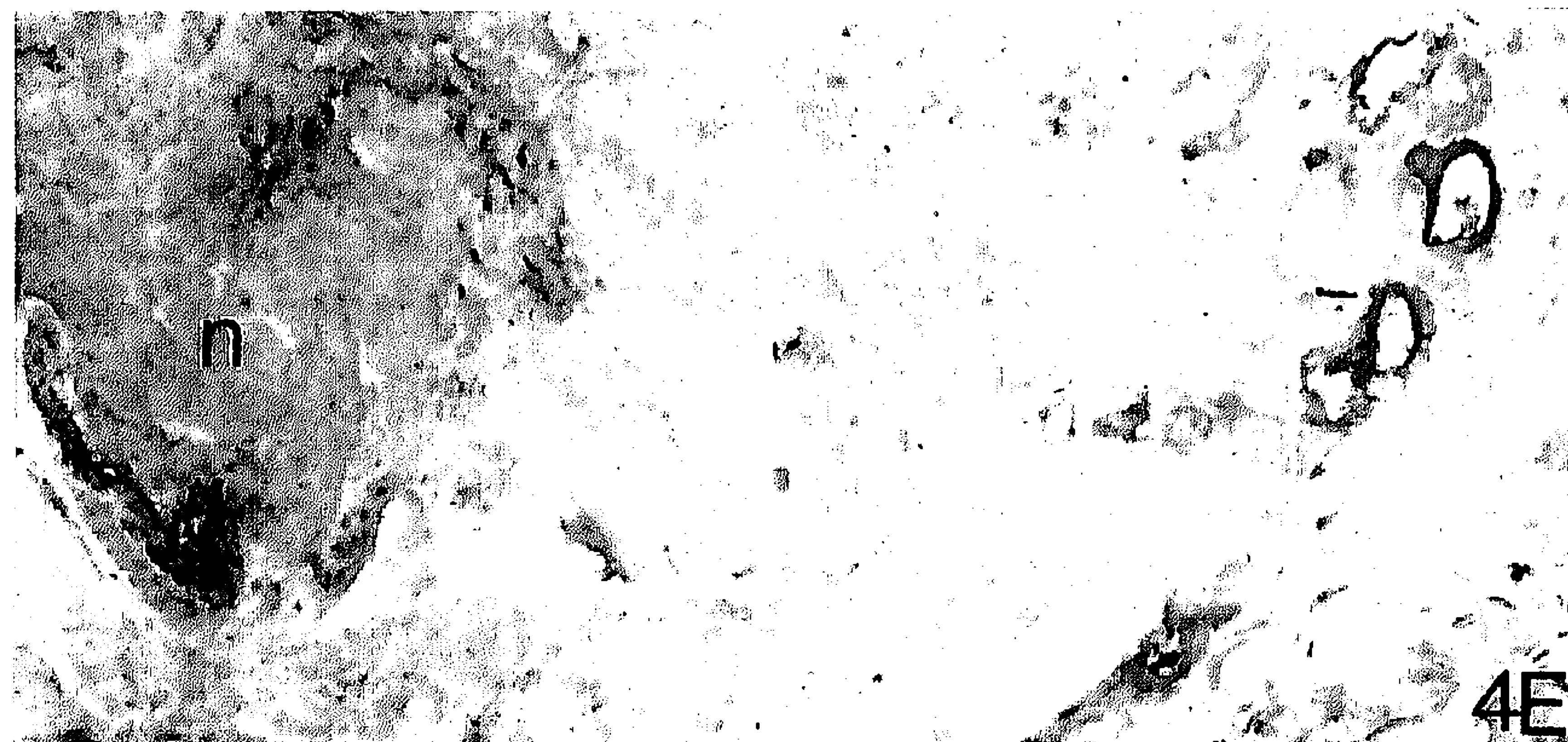
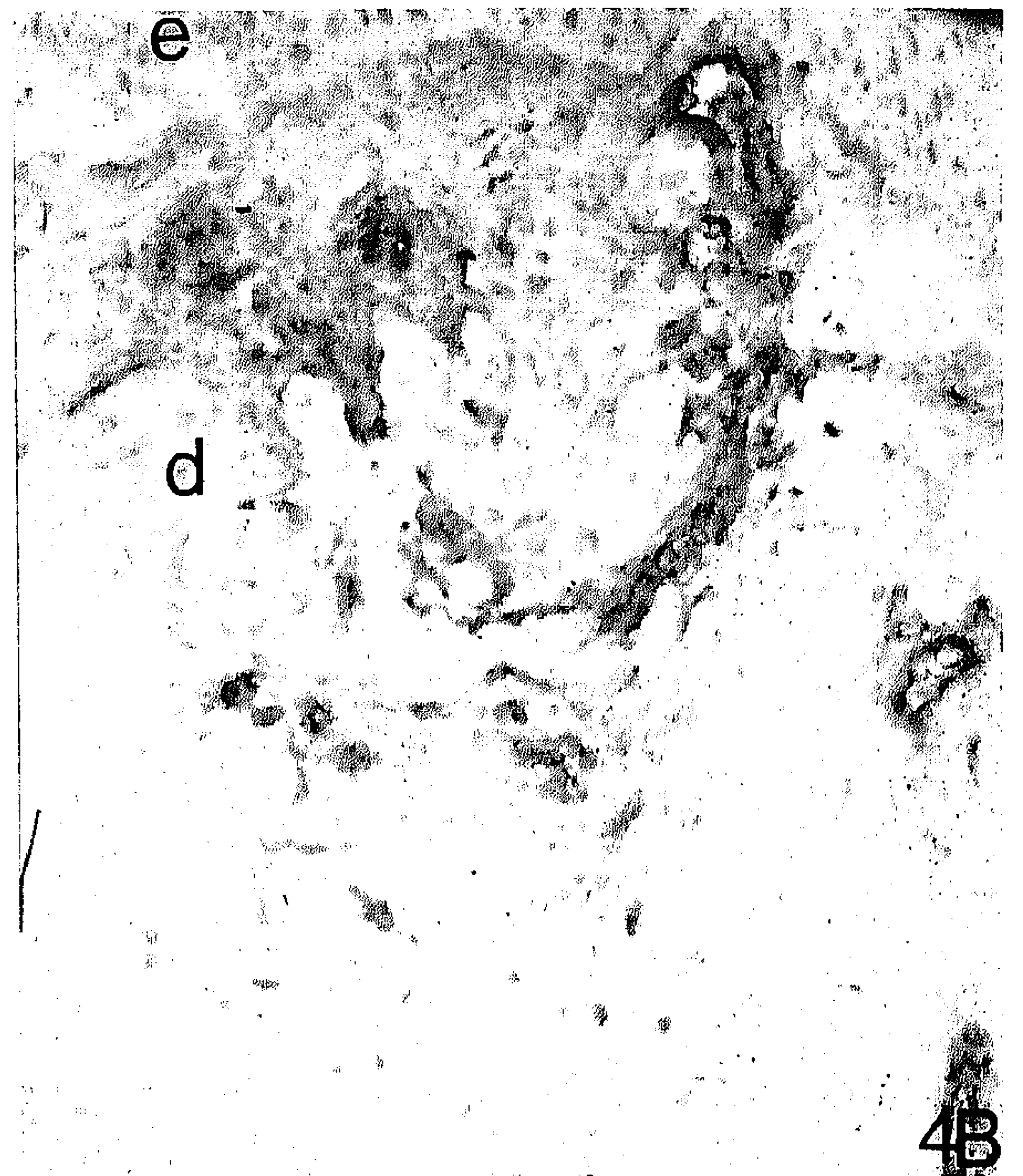
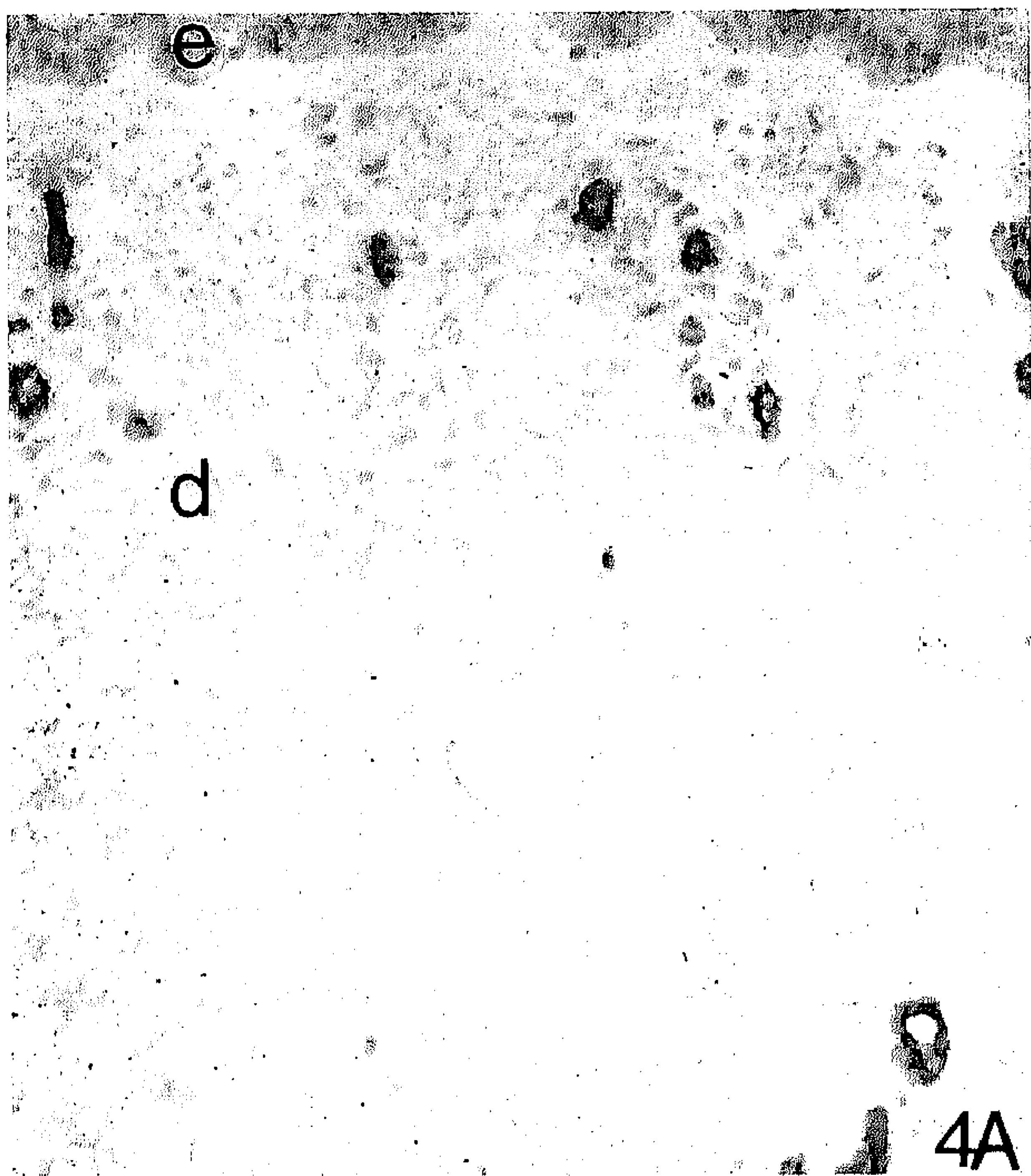
The expression of the above antigens was determined on the microvasculature in the centre of the tumour, at the edge of the tumour, adjacent to the tumour, and within the tumour cells. Staining was performed using an indirect immunoperoxidase procedure, employing amino-ethylcarbazole as chromogen. Sections were counterstained with Harris haematoxylin. The semi-quantitative grading included both intensity (negative: - to highly positive: ++) and estimated percentage of positive cells (score 0 = 0–5 per cent; 1 = 6–25 per cent; 2 = 26–50 per cent; 3 = 51–75 per cent; 4 = 76–100 per cent). The slides were read by two different observers. In cases of disagreement, consensus was reached during joint re-examination.

## RESULTS

An update of the clinical results of the ILP treatment has been recently reported.<sup>8</sup>

The histological changes after various treatment regimes are shown in Fig. 1 (A = before  $\text{IFN}\gamma$  s.c.; B = after  $\text{IFN}\gamma$  s.c. but before ILP), 2 (various time points after ILP with  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , and melphalan), and 3 (after ILP with melphalan alone)

Biopsies prior to  $\text{IFN}\gamma$  s.c. ( $n=6$ ) showed no evidence of necrosis, haemorrhage or fibrin thrombi, although sporadic intravascular thrombocyte aggregation could be detected at the periphery of untreated tumours (Fig. 1A). After  $\text{IFN}\gamma$  s.c., but before ILP with  $\text{TNF}\alpha$  and melphalan, a slight increase in thrombocyte aggregation (three out of nine) occurred (Fig. 1B). Within a period of 24 h after complete treatment, a marked increase in thrombocyte aggregation was observed in all biopsies ( $n=22$ , Fig. 2A). The extent of the aggregates varied from local to a



generalized involvement of the tumour microvasculature, both in the centre and at the periphery of the tumour. This occurred without significant fibrin staining or haemorrhage, although dermal oedema and erythrocytostasis in the microvessels were frequently recorded. After 1 week, the CD41-positive aggregates persisted and extensive necrosis occurred (five out of ten lesions, Fig. 2). Necrosis was of the coagulative type, with or without haemorrhagic elements. This was less obvious in tumours taken from patients who did not develop a complete response. Oedema was frequently recorded and was maintained for several weeks. In one patient, thrombocyte aggregation could still be observed in a biopsy 9 months after perfusion (Fig. 2B). Melanoma lesions ( $n=3$ , Fig. 3) treated with melphalan perfusion alone revealed thrombocyte aggregation but in these patients it was never associated with necrosis.

The sequential immunohistochemical analysis revealed a marked change in the distribution of VWF staining after complete perfusion treatment as compared with the pretreatment biopsies. Prior to  $\text{IFN}\gamma$  s.c., VWF staining was confined to the endothelial layer (+++, 76–100 per cent), both in normal skin and in the melanoma lesions. Half an hour after ILP with  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , and melphalan, a slight increase in perivascular staining was observed in the melanoma lesions (+, 76–100 per cent). Although the VWF staining in the skin outside the melanoma lesion was confined to the endothelial layer, marked subepidermal and perivascular VWF staining was recorded in and above the melanoma lesions 3 h after complete perfusion (Figs 4A and 4B) (+, 76–100 per cent). This was accompanied within a period of 24 h by intravascular thrombocyte aggregation (Figs 4C–4E), erythrocytostasis, and meshwork-like intravascular VWF staining (not shown). In the tumour centre, a decrease of  $\alpha$ -smooth muscle actin (+, 6–25 per cent) and type IV collagen (+, 6–25 per cent) staining in the microvascular walls could be observed. The PAL-E endothelial staining became discontinuous, in comparison with the circular endothelial staining in the peritumoral and untreated tissues (not shown). Tissue factor stain-

ing of the endothelium was negative in all cases, in contrast with the positive staining of the adventitial vascular cells and epidermal and sweat gland epithelial cells in the same sections. Identically treated sections of a squamous cell carcinoma specimen and of normal renal glomeruli were regarded as positive controls for the tissue factor staining procedure.

## DISCUSSION

$\text{TNF}\alpha$  in combination with melphalan and  $\text{IFN}\gamma$ , administered in an isolated limb perfusion setting, has been shown to increase the number of complete remissions and the duration of response in stage IIIA/B melanoma patients as compared with perfusion with melphalan alone.<sup>5</sup> Changes in the microvasculature of the perfused melanoma lesions were observed, especially the upregulation of cellular adhesion molecules.<sup>1</sup> Here we report on the morphological and immunohistochemical analyses of the microvascular coagulative events, with visualization of structural and functional markers of the endothelium.<sup>9</sup>

Sequential biopsies taken before and at various time points after cytokine administration revealed a marked change in the distribution of VWF staining. Before  $\text{IFN}\gamma$  s.c., VWF staining was confined to the endothelial layer. Marked subepidermal and perivascular VWF staining was recorded 3 h after complete perfusion. This was accompanied within a period of 24 h by intravascular thrombocyte aggregation, erythrocytostasis, and intravascular meshwork-like VWF staining. These findings strongly suggest that the tumour microvasculature is the main target in human melanoma in transit metastasis treated with a high dose of rHu- $\text{TNF}\alpha$  in combination with  $\text{IFN}\gamma$  and melphalan. Observations in late biopsies (range to 9 months after perfusion) may indicate that, apart from an acute event, the tumour microvasculature may have undergone long-lasting damage.

Over the last few years, interest in von Willebrand factor (VWF) as a marker of endothelial cell injury has been growing.<sup>10</sup> VWF is

Fig. 4—(A) VWF immunostaining in a frozen section of skin, in the vicinity of a melanoma metastasis, 3 h after perfusion with  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , and melphalan. (e=Epidermis; d=dermis.) (B) VWF immunostaining in a frozen section of skin with melanoma metastasis 3 h after perfusion with  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$ , and melphalan. (e=Epidermis; d=dermis.) (C,D) Double immunostaining for VWF (brown) and CD41 (dark blue) in a frozen section of skin with melanoma metastasis 24 h after perfusion with  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$ , and melphalan. (e=Epidermis; d=dermis.) (E) Double immunostaining for VWF (brown) and CD41 (dark blue) in a frozen section of skin with melanoma metastasis in an area of necrosis, 24 h after perfusion with  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$ , and melphalan. (n=Necrosis)



synthesized by endothelial cells and megakaryocytes and can be stored in endothelial-specific Weibel-Palade bodies<sup>11</sup> and in the  $\alpha$  granules of the platelet. The role of VWF in the adhesion and aggregation process can be looked upon as bridge formation between the thrombocytes and the sub-endothelium of the injured vessel wall.<sup>12-14</sup> Accordingly, VWF can bind with thrombocyte membrane glycoproteins Ib and IIb/IIIa (CD41/CD42) and components of the subendothelium, such as collagen.

TNF and other cytokines may well play a role in the release of VWF by endothelial cells, both *in vitro* and *in vivo*. Alternatively, as stated by Paleolo *et al.*,<sup>15</sup> they might have no direct effect on VWF release but can significantly modulate its acute release in response to thrombin in a complex time- and dose-dependent manner. Van der Poll *et al.* investigated the effect of TNF on the release of VWF into the circulation during a controlled study in six healthy men, sequentially measuring the plasma concentrations of VWF after a bolus intravenous injection of recombinant human TNF $\alpha$  (50  $\mu$ g/m<sup>2</sup>).<sup>16</sup> TNF $\alpha$  induced a marked increase in VWF antigen plasma levels, becoming significant after 45 min and peaking after 4 h. The multimeric organization of circulating VWF was not affected by TNF $\alpha$ . We observed slight perivascular VWF immunostaining within 30 min after ILP (TNF $\alpha$ +melphalan+IFN $\gamma$ ) and pronounced staining at 3 h. Meshwork-like intraluminal VWF staining, erythrosthesis, and thrombocyte aggregations could be observed within a period of 24 h after ILP.

Nawroth *et al.* demonstrated that infusion of low concentrations of TNF $\alpha$  (3  $\mu$ g/animal) into mice bearing meth-A-fibrosarcomas leads to localized fibrin deposition with the formation of occlusive intravascular thrombi in close association with the endothelial cell surface.<sup>7</sup> They proposed a coagulation cascade starting with induction of tissue factor by TNF $\alpha$ . Tissue factor, also known as tissue thromboplastin, is a cell surface glycoprotein responsible for triggering the extrinsic pathway of the coagulation cascade.<sup>17</sup> Induction of tissue factor expression on the endothelium has been reported after *in vitro* incubation with TNF $\alpha$ .<sup>18</sup>

All of these earlier results were found in an experimental setting. Having studied biopsies from patients treated with ILP, however, we were unable to find an increase in fibrin deposition in the tumour vascular bed, or positive immuno-

staining of the endothelial cells for tissue factor. Fixation-related denaturation of tissue factor could not explain the negative staining results in the melanoma lesions, because in the same sections adventitial vascular cells and epidermal and sweat gland epithelial cells stained positive as internal controls. Also, in alignment with the results described by Rao,<sup>23</sup> we found positive staining of identically treated squamous cell carcinoma and normal renal glomeruli.

The increase of perivascular VWF distribution and CD41-positive thrombocyte aggregations associated with the (sub)endothelial cell surface, together with the absence of tissue factor and fibrin staining, indicates that thrombocyte aggregation is not due to local procoagulant activity, but is rather the result of the induced vascular damage or haemostasis. TNF $\alpha$ -induced congestion, haemorrhage, and oedema may represent an impaired blood flow, finally leading to haemorrhagic infarction. Platelet aggregation may enhance congestion and thereby accelerate the occurrence of haemorrhagic infarction. During this process, VWF may play an important role in the adhesion between thrombocytes and the subendothelium. In addition, granulocytes may contribute to the endothelial cell damage. In the current patient group, a significant influx of granulocytes was not consistently observed, in contrast to thrombocyte aggregation. Granulocyte-mediated damage may therefore be of importance, but does not seem to be a *sine qua non*. It can be concluded, however, that all findings support the assumption that the tumour microvasculature is an important target for TNF $\alpha$  triple combination therapy.

#### ACKNOWLEDGEMENT

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