Transpl Int (1993) 6: 251–257

Transplant International

Effect of platelet-activating factor (PAF) receptor blockers on smooth muscle cell replication in vitro and allograft arteriosclerosis in vivo

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Received: 12 June 1992/Received after revision: 3 December 1992/Accepted: 5 January 1993

Abstract. Platelet-activating factor (PAF) stimulates smooth muscle cell (SMC) replication both in vivo and in vitro. In this study we have investigated whether PAF receptor-blocking molecules modulate SMC replication in vitro and the generation of allograft arteriosclerosis in vivo. SMC cultures were established from baby rat aorta media and fibroblast control cultures from the adventitia. Identification of the cultured cell types was determined both by immunohistochemistry and electron microscopy. Both cell types replicated in culture with 10% fetal calf serum (FCS). The addition of PAF-C_{18} enhanced, and the addition of three PAF receptor inhibitors – WEB 2086, WEB 2170, and BN 50739 – reduced, SMC replication and protein synthesis in a dose-dependent fashion in vitro until toxic concentrations were reached. The most potent of these drugs, WEB 2170, was then delivered at the rate of 12 mg/kg per day to recipients of rat aortic allografts. The responses were quantitated by autoradiography after short-term labeling of the recipients with tritium-labeled thymidine (^{3}H-TdR) and by quantitative morphology. Administration of the PAF receptor blocker had no impact on the replication of the inflammatory cells in the allograft adventitia nor on the replication of SMCs in the media and intima. Administration of the PAF receptor blocker delayed the generation of allograft arteriosclerosis slightly, but not significantly. These results suggest that PAF is not an essential component in the inflammatory cascade leading to allograft arteriosclerosis.

Key words: PAF, allograft arteriosclerosis – Allograft arteriosclerosis, PAF receptor blockers – Smooth muscle cell replication, PAF receptor blockers

Introduction

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine; PAF) is a vasoactive ether lipid [4, 9, 13]. The molecule is synthesized by many types of activated blood cells, including macrophages [2, 8]. Endothelial cells also synthesize PAF, but most of the material remains associated with the endothelial surface [7]. In addition, endothelial cells can take up and metabolize PAF from the extracellular fluid [3, 11].

Platelet-derived growth factor (PDGF), particularly the BB isomer [12], is a strong stimulant to smooth muscle cell (SMC) replication in vitro. Recent experiments [10] suggest that PAF may also stimulate the proliferation of cultured SMCs and that this effect is additive to the proliferative effect of PDGF.

Macrophages are an integral component, together with T- and B-lymphoid cells, in the perivascular inflammatory infiltrate of chronic rejection [5]. It was therefore of interest to investigate whether PAF receptor blockers are inhibitors of SMC replication in vitro, and whether they, when administrated in vivo, inhibit allograft arteriosclerosis, which is characteristic of chronic rejection.

Materials and methods

Experimental animals

Inbred WF (AGB2-RT1^v) and DA (AGB4-RT1^a) rat strains were used for the transplantations. All animals were purchased from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). Male rats weighing 200–300 g and 2–4 months of age were used as donors and recipients.

SMC and fibroblast cultures

SMCs and fibroblasts were isolated from the aortas of 9- to 11-day-old DA rats. A modification of the method by Thyberg et al. [12] was used. The aortas were opened longitudinally and the intimal layer was gently scraped off; the media and the adventitia were carefully separated and sliced into small pieces. The different layers were digested with 0.1% collagenase and 0.02% DNAse in phosphate-buffered saline (PBS) at +37 °C for 1–2 h. Isolated cells from the media and the adventitia (SMC and fibroblasts, respectively) were centrifuged, suspended in culture medium [Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS), Sera-Lab, Sussex, UK; 2 μmol/ml glutamine; 100 μg/ml streptomycin; and

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Table 1. Immunohistochemical staining of cultured smooth muscle cells (SMC) from allograft media, cultured fibroblasts (FIBR) from allograft adventitia, and A10-cells. n.d., Not done

Antibody	SMC	FIBR	A10
Smooth muscle cell α-actin	+ + + +	(+)[a]	+ +
HHF-35	+ +	+	+
CGA-7	+/−	−	−
Desmin	+	−	n.d.
Vimentin	+	+ + +	n.d.

[a] Occasional granular staining

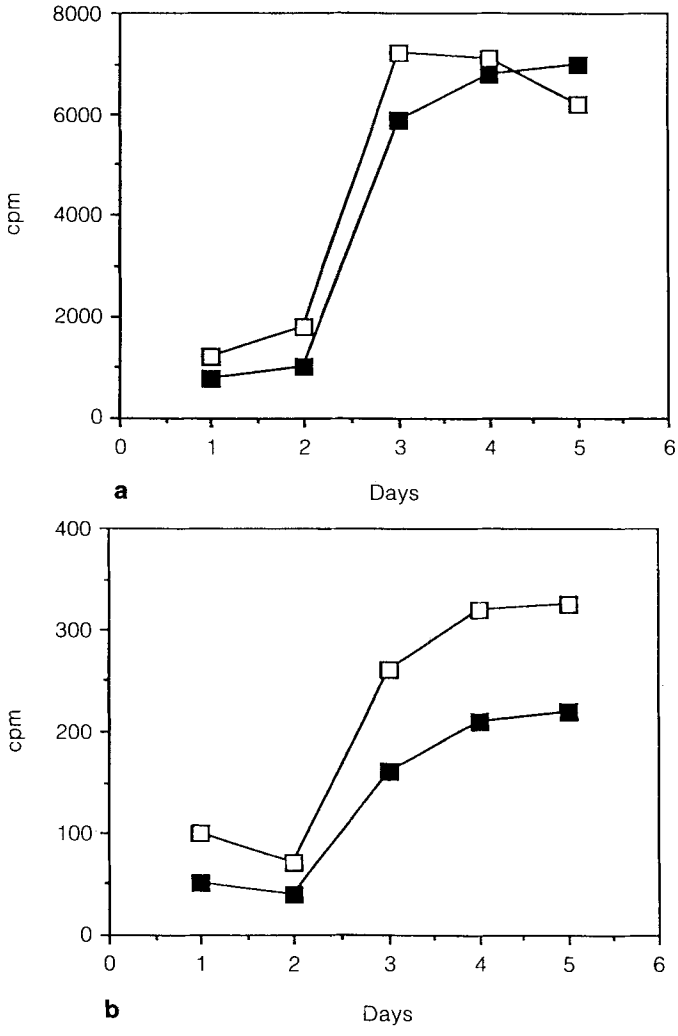

Fig. 1. **a** ^{3}H-thymidine and **b** ^{3}H-glycine incorporation of cultured smooth muscle cells (■) and fibroblasts (□) derived from 9- to 11-day-old DA rat aortas on days 1–6; *cpm*, counts per minute

100 U/ml penicillin], seeded in plastic flasks, and incubated in 5% CO_2 at + 37 °C. A cell line, A10, derived from rat aortic media, was used for control.

Assays in vitro

SMC and fibroblasts were seeded in 96-well multidishes (8000 cells/well) in full culture medium, supplemented with 10% FCS. The drugs, PAF-C_{18} (C_{18} analogue of 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; Novabiochem, Läyfelfingen, Switzerland), BN 50739 (Henri Beaufour Institute, Paris, France), WEB 2086 or WEB 2170 (Boehringer-Ingelheim, Ingelheim-am-Rhein, Germany) were added to the medium in different concentrations (0–10 μg/ml). The cells were exposed daily to 1 μCi/ml of tritium-labeled thymidine (^{3}H-TdR; NEN Chemicals, Boston, Mass., USA) or tritium-labeled glycine (^{3}H-glycine) for 24 h to quantitate DNA and protein synthesis, respectively. After 24 h, the wells were washed three times with PBS, detached with 1.25% trypsin, and mixed with OptiPhase "HiSafe" 3 (LKB-Wallac, Turku, Finland). The radioactivity was measured with a Rackbeta liquid scintillation counter (LKB-Wallac). To assess the viability of cultured cells and to exclude any toxic effect of the inhibitory drugs on SMC in vitro, the medium was removed from additional cultures on day 3 and replaced with conditioned medium from SMC bulk cultures.

Aortic transplantations

The DA-to-WF strain combination was used for allografts, and syngeneic controls were made from the DA-to-DA strain. The experimental animals were anesthetized with intraperitoneal chloral hydrate (6 ml/kg). A segment of the descending thoracic aorta, approximately 3 cm in length, was excised, perfused with saline, and used as a transplant [5]. The graft was transplanted in heterotopic position below the renal arteries and above the bifurcation forming a "loop" in the abdominal cavity of the recipient. This modification of the original technique allowed us to obtain material for histological and biochemical determinations. The histological results of the modified graft did not differ from those originally reported [5]. The cranial suture line was made as close to the renal arteries as technically possible to minimize the difference in diameter. End-to-end anastomosis was performed using 9-0 continuous nylon suture. The grafts were removed at 10 and 20 days and at 1, 2, 3, 5, and 6 months after transplantation and they were processed for histology, autoradiography, frozen section immunohistochemistry, and biochemical determinations. Both the in situ fixation, done by infusion of 10% neutral formaldehyde to the left atrium prior to sacrifice of the anesthetized animal, and regular fixation were employed with similar results. Specimens for immunohistochemistry were immersed in Tissue-Tek (Miles, Elkhard, Ind., USA) and snap-frozen.

WEB 2170 was given to the recipients in drinking water at a dose rate of 12 mg/kg per day. According to the manufacturer's instructions, this dose is twice the ED_{50} that protects the rat from PAF-induced lethality.

Histological specimens and staining

For evaluation of morphological changes, paraffin sections were stained with Mayer's hematoxylin and eosin (HE) and Orcein for elastic fibers. Longitudinal sections were also prepared, where the vessel wall could be observed both on the graft and the host side of the suture line, as well as cross-sections for quantitation of circular changes in the graft. Quantitative histology (morphometry) was always done from the middle (1/3) section of the transplant. Aortas from nontransplanted rats and thoracic aortas from the recipient rats were used as controls.

Immunohistochemistry

For immunohistochemistry, 3- to 4-μm-thick frozen sections were stained by the immunoperoxidase (IP) technique using monoclonal antibodies to alpha SMC actin (Bio-Markor, Rehovot, Israel), to alpha and gamma actin recognizing skeletal, cardiac and SMCs (HHF35; Enzo Diagnostics, Syosset, NY, USA); to alpha and gamma actin recognizing SMCs only (CGA7; Enzo Diagnostics), to desmin (DAKO, Glostrup, Denmark), and to vimentin (DAKO).

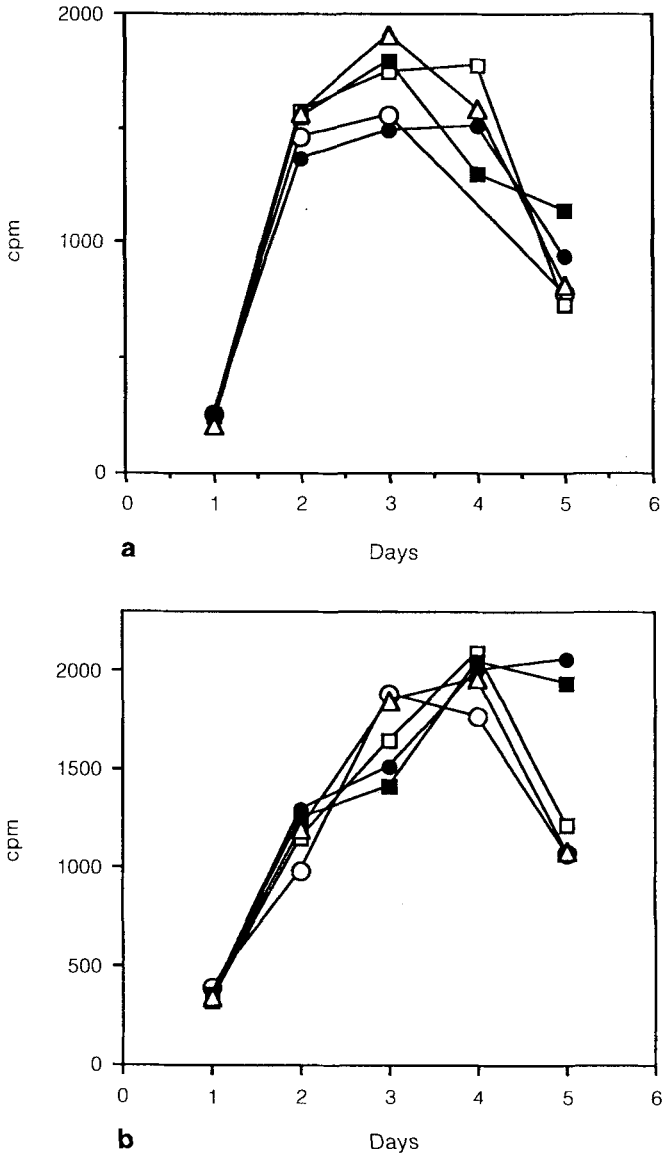

Fig. 2a,b. The effect of PAF-C_{18} on the replication of cultured smooth muscle cells expressed as **a** ^{3}H-thymidine and **b** ^{3}H-glycine incorporation in vitro. ○ Control; ● 10 μg/ml; □ 1 μg/ml; ■ 0.1 μg/ml; △ 0.01 μg/ml; *cpm*, counts per minute

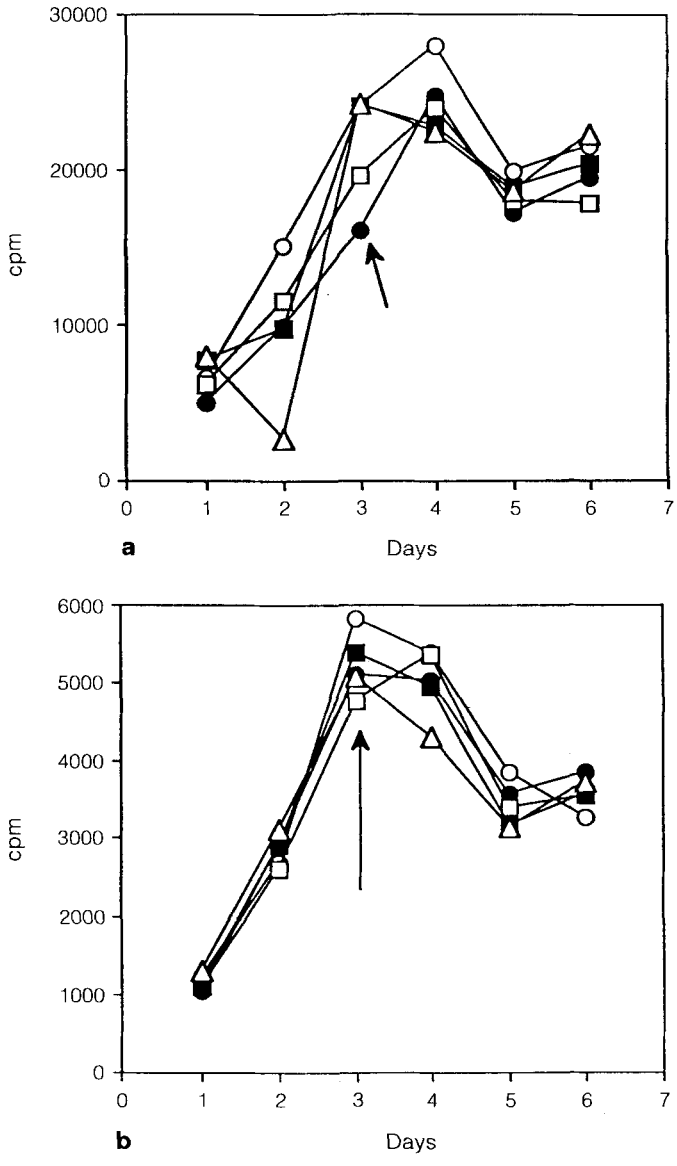

Fig. 3a,b. The effect of WEB 2086 on the replication of cultured smooth muscle cells expressed as **a** ^{3}H-thymidine and **b** ^{3}H-glycine incorporation on days 1–6. *Arrow* indicates the time when conditioned media were added. ○ Control; ● 10 μg/ml; □ 1 μg/ml; ■ 0.1 μg/ml; △ 0.01 μg/ml; *cpm*, counts per minute

The cryosections were stained using the two-layer indirect IP technique described earlier [5]. Briefly, the sections were incubated with an appropriately diluted monoclonal antibody (usually 1:20; alpha actin 1:1000), washed, and consecutively incubated with peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig (Dakopatts, Denmark), followed by treatment with a substrate solution containing chromogen 3-amino-9-ethylcarbazole (AEC). Hydrogen peroxide was added to the AEC solution immediately prior to use. The samples were counterstained with Mayer's hemalum solution and mounted.

Histological quantitation of rat aortic transplants

The morphological changes were quantitated according to standard morphometric principles [1] and expressed as point score units (psu), i.e., the mean number of points falling over a given anatomical area using straight cross-sectional lines and a 0.02-mm grid. The following variables were evaluated: the number of nuclei and the thickness of different layers of aorta (adventitia, media, and intima) separated from each other by internal and external elastic laminae. A minimum of five technically successful transplantations was used for each time point if not otherwise indicated, and their means (± SEM) were used as final scores.

Autoradiography

Some of the rats received 300 μCi of ^{3}H-TdR by IV injection 3 h before sacrifice. Histology was processed from paraffin sections, emulsion autoradiography (Ilford L.4; Ilford, Cheshire, UK) was performed, and the labeling of the nuclei in the transplanted aortic wall was compared to the labeling index in the recipient's own aorta. In some cases, autoradiography was performed on immunohistochemically stained slides to identify the types of cells incorporating ^{3}H-TdR. The results were quantitated as number of labeled nuclei/layer, i.e., adventitia, media, and intima, using cross-sectional areas of the aorta.

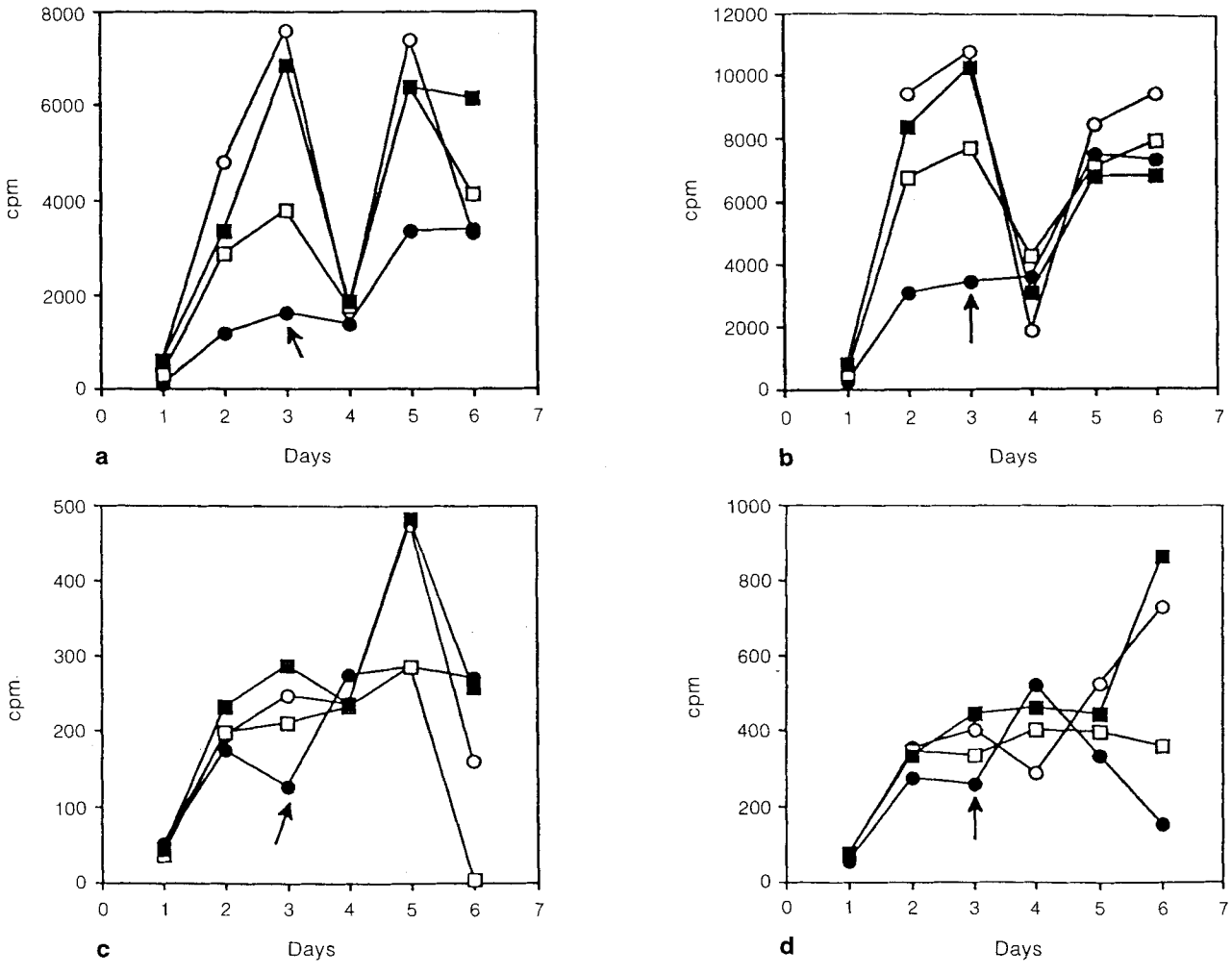

Fig. 4a–d. The effect of WEB 2170 on the replication of: **a** cultured smooth muscle cells expressed as ^{3}H-thymidine **b** fibroblasts expressed as ^{3}H-thymidine **c** SMCs expressed as ^{3}H-glycine **d** FIB expressed as ^{3}H-glycine incorporation on days 1–6. *Arrow* indicates time when conditioned media were added. ○ Control; ● 0.1 μg/ml; □ 0.03 μg/ml; ■ 0.01 μg/ml; *cpm*, counts per minute

Results

Effect of PAF-C_{18}, WEB 2086, WEB 2170, and BN 50739 on SMC replication in vitro

SMC cultures were established from baby rat aorta media and cultured in flasks until confluent. For control, fibroblast cultures were established from the vascular adventitia.

The cell lines were investigated at the beginning of the secondary culture for the presence of SMC markers (alpha-actin) and by electron microscopy to demonstrate myofibrils in cell cytoplasm. As seen in Table 1, cultures established fron the media retained, to a large extent, the immunohistochemically demonstrable alpha-actin and gamma-actin as compared to the A10 cell line, which served as a control for muscle cell differentiation. Electron microscopy (not shown) showed these cells to be largely of the "secretory" phenotype. Myofibrils were seen in the cell cytoplasm, though in smaller numbers. In cultures initiated from the adventitia, practically no actin or desmin reactivity was present in immunocytochemistry, and no myofibrils were recorded in the cytoplasm. In electron microscopy, the adventitia-derived cells were indistinguishable from ordinary fibroblasts. In culture medium with 10% FCS containing ample amounts of growth factors, both cell types proliferated optimally, although the fibroblast lines reached confluency 2–3 days earlier than the SMC lines (Fig. 1).

In the first experiment, we reinvestigated the effect of PAF on the replication of SMC in vitro. PAF-C_{18} was added to the cultures at concentrations ranging from 0.01 μg/ml to 10 μg/ml, and the incorporation of ^{3}H-TdR (for DNA synthesis) or ^{3}H-glycine (for protein synthesis) was used to quantitate the proliferative responses. As seen in Fig. 2, the addition of PAF-C_{18} slightly stimulated the SMC replication rate in vitro. The stimulatory effect was not dose-dependent, but responses of approximately 20% above the control level were recorded with all except the highest test concentration.

In the second experiment, WEB 2086 was added to the culture medium at the initiation of the culture using the concentration range of 0.01 μg/ml–10 μg/ml, and the incorporation of either ^{3}H-TdR or ^{3}H-glycine was used to

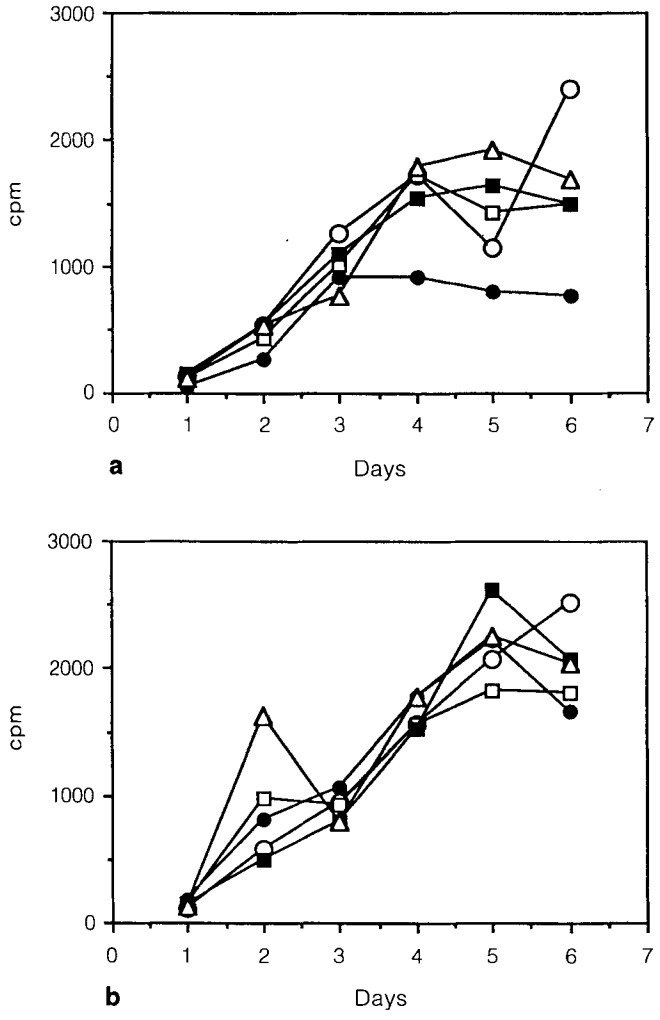

Fig. 5 a, b. The effect of BN 50739 on the replication of cultured smooth muscle cell expressed as **a** ^{3}H-thymidine and **b** ^{3}H-glycine incorporation in vitro. ○ Control; ● 10 μg/ml; □ 1 μg/ml; ■ 0.1 μg/ml; △ 0.01 μg/ml; *cpm*, counts per minute

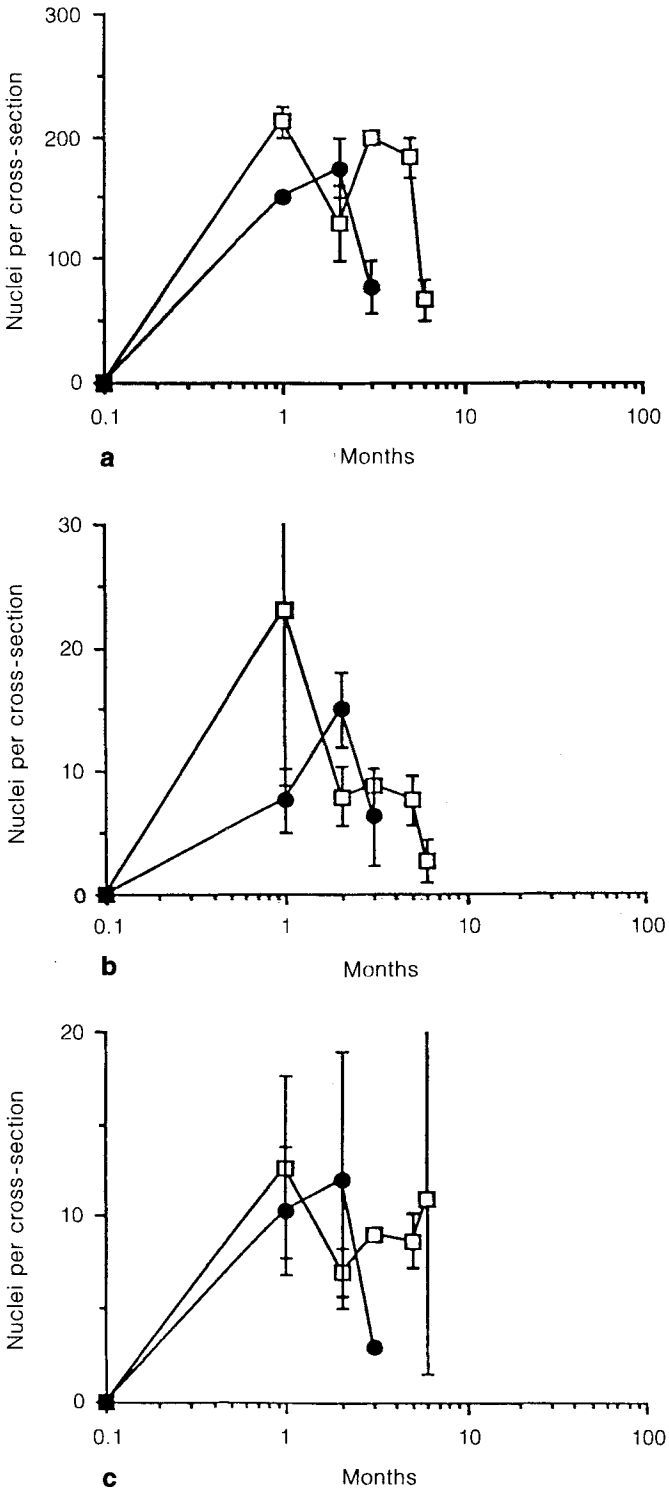

Fig. 6 a–c. Frequency of labeled cells per cross-section expressed as point score units (psu) in the **a** adventitia, **b** media, and **c** intima of aortic allografts when the recipients were treated with 12 mg/kg per day of WEB 2170 (●) or were left untreated (□). Two to seven determinations were made at each time point where SEM is indicated

quantitate the proliferative responses, as previously described. On day 3 of culture, the drug-containing medium was replaced with conditioned medium without the drug to ascertain the viability of the cells in culture and the reversibility of the effect. As seen in Fig. 3, WEB 2086 inhibited the SMC replication over a large concentration area in a dose-dependent fashion, although the inhibitory effect was not particularly pronounced, being at the most 25%–30% of the control at the highest test concentration. The proliferation peaked on day 4, whereafter it declined if no conditioned medium was added (not shown). If conditioned medium was added, the decline was followed by another proliferative burst. The inhibitory effect of WEB 2086 proved to be reversible and was not toxic to the cells even at the highest test concentration of the drug. The inhibitory effect on protein synthesis was even less pronounced.

In the third experiment, WEB 2170 was used under similar conditions (Fig. 4). Here, the inhibitory effect of the drug was more pronounced and more clearly concentration-dependent: at the second highest test concentration the inhibitory effect was 50% of the control. The peak response was observed, again, on day 3 of culture. If conditioned medium was added, it was followed by a second burst of replication. The second burst indicated that the inhibitory effect of WEB 2170 was reversible and that in the highest test

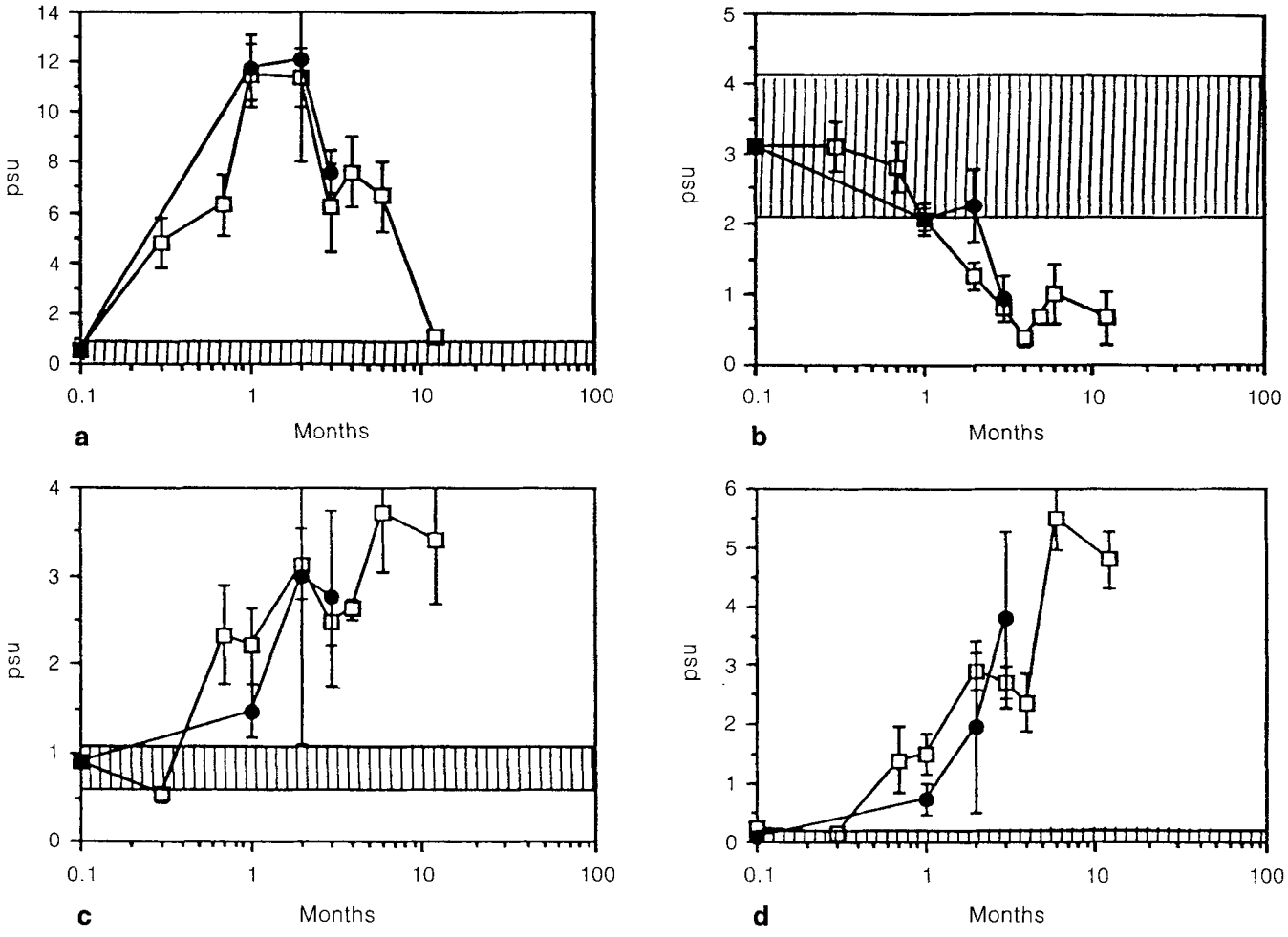

Fig. 7a–d. Nuclear densities expressed as point score units (psu) of **a** adventitia, **b** media and **c** intima of aortic allografts, and **d** intima thickness when the recipients were treated with 12 mg/kg per day of WEB 2170 (●) or were left untreated (□). Three to seven determinations were made at each time point where SEM is indicated. *Shaded box* indicates normal DA control aorta ± SD

concentration, 0.1 µg/ml, the drug was obviously toxic to the SMC.

To investigate the specificity of the inhibitory effect, a fourth experiment was performed with adventitial fibroblasts (Fig. 4). Again, a similar inhibitory effect was observed, indicating that the effect of WEB 2170 was nonspecific. The second burst of replication after addition of the condition medium suggested that the fibroblast cultures were less sensitive to the toxic effect of this compound. The inhibitory effect of WEB 2170 on protein synthesis in both SMC and fibroblast cultures was only slightly weaker than on thymidine uptake.

In the fifth and final in vitro experiment, a third inhibitor, BN 50739, was used under similar conditions. This time, only a slight inhibitory effect was recorded, which was not even clearly dose-dependent (Fig. 5). The inhibitory effect of BN 50739 was smaller than that of WEB 2170 and of the same order of magnitude as the effect of WEB 2086.

Effect of WEB 2170 on SMC replication in vivo and on allograft arteriosclerosis

Of these four compounds we selected WEB 2170 as the most promising one for further in vivo studies.

Aortic transplantations were performed from the DA-to-WF strain. The drug was administered in drinking water (forced administration) at a dose rate of 12 mg/kg per day, which is twice the ED_{50} dose of PAF-induced lethality in the rat.

The effect was quantitated in two different ways: first, by pulse labeling with 300 µCi of ^{3}H-TdR for 3 h prior to sacrifice, followed by autoradiography and, second, by quantitative histology from HE-stained histological specimens.

As seen in Fig. 6, in nontreated recipients, autoradiography showed a strong proliferative response from the allograft adventitia. We have already shown that most of the proliferating cells in the adventitia represent inflammatory cells [6]. Administration of WEB 2170 did not inhibit adventitial inflammatory cell proliferation.

There was also an early burst of proliferating cells in the media that gradually declined, and a similar proliferative response was observed in the intima of nontreated allograft recipients. We have previously demonstrated that most of the proliferating cells in the allograft media and intima represent SMC. Administration of WEB 2170

did not inhibit the proliferative response of SMC either in the media or in the intima.

In Fig. 7, the aortic allograft histology has been quantitated by morphometry from histological specimens. In nontreated allografts there was a rapid accumulation of inflammatory white cell nuclei in the adventitia, peaking at 1–2 months post-transplantation, whereafter the response declined. In the media there was a gradual loss of SMC nuclei, indicating media necrosis. The number of intimal nuclei began to increase after the 1st month post-transplantation and, concomitantly, there was an increase in the intimal thickness, which continued to the end of the experiment, 11 months after transplantation. Administration of WEB 2170 had no effect on the intensity of adventitial inflammation, nor on the loss of nuclei in the media, and had only a small effect, or none at all, on the generation of intimal arteriosclerosis.

Discussion

In a normal vessel, the intact endothelium forms an effective barrier preventing the entry of PAF from the circulation into the interstitial space, thus shielding the underlying SMC from its effects. We assume, however, that during the generation of arteriosclerosis the vascular endothelium is damaged, resulting in a release of PAF into the interstitial space. More important, however, is the fact that macrophages in the allograft adventitia may provide an additional source of PAF which, in turn, may be responsible for the induction of SMC proliferation and their migration into the intima.

We have recently demonstrated (in preparation) that extracts of aortic allografts contain vastly increased quantities of epidermal growth factor (EGF), platelet-derived growth factor beta (PGDFbb), and insulin-like growth factor 1 (IGF-1), all of which have previously been linked to SMC replication in vitro [12]. In the absence of a suitable RIA assay for PAF, we were unable to document the presence of PAF in the interstitial fluid.

These experiments provide evidence, albeit indirect, that PAF is not an essential mediator in the generation of allograft arteriosclerosis. The in vitro experiments demonstrated some enhancement in the SMC replication rate with PAF-C_{18} and inhibition of SMC replication by PAF receptor blockers, which was obviously nonspecific and also demonstrable with fibroblast lines. On the other hand, we were unable to modify the arteriosclerotic process in vivo with the more effective one of these two molecules.

We have previously suggested that persistent perivascular inflammation, observed in all allografts during chronic rejection, is the driving force for arteriosclerotic changes in the arteries of these transplants. We also think that the effect is mediated not only by cytokines (such as IL-1) but also by lipid mediators of inflammation (such as thromboxane A2) derived from the platelets and inflammatory cells. The action of these proinflammatory molecules may lead to damage of the graft vascular endothelium which, in turn, may provide growth factors that are the final molecules responsible for SMC replication in the vascular wall.

These results clearly suggest that PAF is not an integral part of the molecular cascade leading to intimal thickening in chronic rejection.

Acknowledgements. This work was supported by grants from the University of Helsinki, the Sigrid Juselius Foundation, the Finnish Academy, the Finnish Medical Society Duodecim, the Kidney Foundation, and the Finnish Cultural Foundation, Helsinki, Finland, as well as the Research and Science Foundation of Farmos, Turku, Finland.

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