Kidney International, Vol. 52 (1997), pp. 1478–1485

HORMONES - CYTOKINES - SIGNALING

Leukocyte-polytetrafluoroethylene interaction enhances proliferation of vascular smooth muscle cells via tumor necrosis factor-α secretion

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Leukocyte-polytetrafluoroethylene interaction enhances proliferation of vascular smooth muscle cells via tumor necrosis factor-α secretion. Intimal hyperplasia of vascular smooth muscle cells (VSMC) at the venous anastomosis of arteriovenous grafts represents the most common cause of vascular access failure in hemodialysis patients. Upstream release of growth factors from leukocytes activated by adhesion to the graft material may play a role in this lesion. We evaluated the effect of interaction of peripheral blood mononuclear cells (PBMC) with polytetrafluoroethylene (PTFE) on proliferation of VSMC. Vascular smooth muscle cell proliferation was significantly increased by conditioned media from human PBMC incubated with PTFE. Peripheral blood mononuclear cell adhesion to PTFE could not be antagonized by the β_1 integrin ligand-containing peptide GRGDSP, but was attenuated by EDTA consistent with β_2 integrin-mediated adhesion. Soluble scavenger receptor ligands at high concentrations had no effect on adhesion to PTFE excluding any contributory role of scavenger receptors in this interaction. Neutralizing antibodies to TNF-α significantly attenuated the mitogenic effect of PBMC/PTFE conditioned media and a marked increase in TNF-α secretion by PBMC on PTFE was detected by ELISA. These studies demonstrate that PBMC interaction with PTFE can promote proliferation of VSMC via increased production of TNF-α and perhaps other cytokines. Leukocyte interaction with PTFE causing enhanced secretion of TNF-α and consequent VSMC proliferation may account for the development of venous intimal hyperplasia in hemodialysis patients with arteriovenous grafts.

Angioaccess failure is a major source of morbidity in hemodialysis patients and accounts for at least 15% of their hospitalizations [1–3]. The majority of access failure occurs in patients with arteriovenous grafts made of polytetrafluoroethylene (PTFE), which represent the most common form of angioaccess in hemodialysis patients in the United States [4–10]. Arteriovenous graft revision has a high failure rate and is expensive [2]. Although some studies have suggested hypercoagulable states as a cause of angioaccess failure [11], this hypothesis has two weaknesses: (*1*) anticoagulation is unable to prevent arteriovenous graft failure in a majority of patients and (*2*) the most common cause of PTFE arteriovenous graft failure is not hypercoagulable states, but instead intimal hyperplasia at the venous anastomosis [5, 12]. Hence, graft thrombosis in the majority of patients is due to venous outflow stenosis that eventually diminishes blood flow sufficiently to allow thrombosis to occur. However, the pathogenesis of this lesion in hemodialysis patients with PTFE grafts is not well understood, as reviewed at length elsewhere [5]. Pathologically, the lesion has been shown to consist of vascular smooth muscle cell (VSMC) proliferation with consequent lumenal narrowing [12]. Hemodynamic forces have been postulated to cause this lesion in patients with arteriovenous grafts, presumably as a consequence of turbulence at the venous anastomosis. Arteriovenous fistulas have high turbulence as well and develop stenosis at venous bifurcations, where turbulence is maximal. These findings suggest a role for turbulence in the development of intimal hyperplasia, although the incidence of fistula stenosis is substantially less than in grafts, and reducing turbulence by changing from end-to-side to end-to-end anastomosis has unfortunately not decreased the incidence of intimal hyperplasia [7]. An additional hypothesis is that there may be upstream release of growth factors released by platelets aggregating at needle puncture sites. Although needle puncture is common to both fistulas and grafts, and while grafts that have never been used can develop venous intimal hyperplasia as well, this tends to occur at a much slower rate and hence a role for platelets in intimal hyperplasia is certainly plausible [5]. As Swedberg et al have pointed out, this lesion can also occur in the distal anastomosis of arterial bypass grafts (in which there is no needle puncture) [12]. While undoubtedly these variables all could contribute to intimal hyperplasia, they do not clearly account for the disproportionate failure of arteriovenous grafts, suggesting that one or more factors related to the graft material itself may play a role.

Activated leukocytes are an important source of VSMC growth factors such as tumor necrosis factor-alpha (TNF-α), a cytokine strongly implicated in causing VSMC proliferation in vein grafts, arterial balloon injury and acute cardiac rejection among other clinical settings [13–15]. Other investigators have shown that peripheral blood leukocytes and other cells can adhere to PTFE with a variety of biological responses [16–19]. PTFE arteriovenous grafts do not endothelialize in humans; hence, it is plausible that circulating leukocytes may adhere to the graft

Key words: hemodialysis, leukocytes, polytetrafluoroethylene, tumor necrosis factor-α, smooth muscle cells.

Received for publication March 31, 1997
and in revised form July 14, 1997
Accepted for publication July 14, 1997

material and release cytokines upstream of the venous anastomosis, exposing VSMC to growth factors and resulting in intimal hyperplasia at the venous anastomosis of the graft, though this hypothesis has not been tested. In the present study we have evaluated the mechanism by which the interaction between human peripheral blood mononuclear cells (PBMC) and PTFE used in arteriovenous grafts may modulate VSMC proliferation.

METHODS

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using standard methodology [20]. In brief, whole blood was collected in 2.7% EDTA in a 10:1 (vol/vol) ratio and mixed 1:1 (vol/vol) with Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY, USA). The solution was layered carefully over 15 ml of Ficoll-Paque (Pharmacia) in a 50 ml polypropylene tube and centrifuged at 2,200 rpm for 20 minutes. The interface was collected, diluted with HBSS and centrifuged at 1,500 rpm for 10 minutes at 4°C. The pellet was resuspended in centrifuged plasma and centrifuged again at 1,200 rpm for five minutes at 4°C. Cells were then washed three additional times with HBSS and then resuspended at 10^6/ml in serum-free Dulbecco's MEM (DMEM, GIBCO) containing penicillin 50 U/ml and streptomycin 50 μg/ml for use in experiments. Trypan blue exclusion studies demonstrated > 95% of PBMC to be viable.

Incubation of peripheral blood mononuclear cells and preparation of conditioned media

Wells on 96-well plastic culture plates (Becton-Dickinson, Lincoln Park, NJ, USA) were coated with 50 μl/well of a solution of polyhydroxyethylmethacrylate (polyHEMA, 1.2 g in 10 ml of 95% ethanol; Sigma Chemical Co., St. Louis, MO, USA) and dried under a laminar flow hood for 48 hours to produce a translucent surface, which other investigators have shown does not allow cell adhesion [21]. The PTFE graft material (Goretex®) was generously provided by W.L. Gore and Associates, Inc. The material was cut into discs and carefully secured into the bottom of selected polyHEMA coated wells with the remaining polyHEMA wells serving as control. Therefore, cells in wells coated with the nonadhesive substrate polyHEMA would mimic circulating leukocytes, while cells in wells coated with polyHEMA plus a disc of PTFE would mimic leukocytes interacting with the PTFE graft material. Suspensions of PBMC at 10^6/ml in serum-free DMEM were added to all wells. Plates were incubated at 37°C in a 95% air 5% CO_2 environment for 24 hours. The supernatants were then collected, centrifuged at 1,500 rpm for 10 minutes and then filtered using a 0.2 μm pore diameter syringe filter (Acrodisc; Gelman Corp, Ann Arbor, MI, USA). Absence of cells in the conditioned media (CM) was confirmed by examination with a hemacytometer. There was no adhesion of PBMC to polyHEMA. Conditioned media were used either immediately for experiments or were stored frozen in aliquots at −70°C and defrosted immediately before use.

Vascular smooth muscle cell proliferation studies

Rat aortic smooth muscle cells (herein termed VSMC) were obtained from American Type Culture Collection (Rockville, MD, USA; A-10 1476-CRL) and grown in DMEM containing 50 U/ml penicillin, 50 μg/ml streptomycin and 20% nonheat-inactivated fetal calf serum (FCS; GIBCO) at 37°C in a 95% air, 5% CO_2 environment. For experiments VSMC were detached by washing with PBS followed by brief exposure to 0.25% trypsin/EDTA solution. Cells were seeded onto 96-well tissue culture plates (Becton Dickinson) at 5×10^3/ml in DMEM containing 2% nonheat-inactivated FCS and incubated at 37°C for six hours to allow attachment. The media was then removed and replaced with DMEM containing 2% nonheat-inactivated FCS alone or 2% nonheat-inactivated FCS plus CM from PBMC incubated on polyHEMA alone or polyHEMA/PTFE at varying concentrations. After 72 hours cells were washed with phosphate-buffered saline, detached using 0.25% trypsin/EDTA solution and counted using a hemacytometer.

Additionally, to determine whether uremic sera might modulate the VSMC response to PBMC/PTFE CM, VSMC proliferation studies with PBMC/polyHEMA and PBMC/PTFE CM were carried out using non-heat inactivated sera collected predialysis and pooled from three patients on chronic hemodialysis at 2% concentration in place of FCS.

Vascular smooth muscle cell proliferation studies using other conditioned media

Additional studies were carried out using the macrophage cell line J774.16 to provide a comparison with PBMC. Conditioned media were prepared from J774.16 macrophages incubated on plates coated with polyHEMA alone or polyHEMA-coated plates containing discs of PTFE in identical fashion as for PBMC. VSMC proliferation studies were then carried out as described above.

Peripheral blood mononuclear cell adhesion studies and role of integrins and scavenger receptors

Adhesion studies were carried out as described previously with modification [22]. Ninety-six-well tissue culture plates were coated with polyHEMA and selected wells had discs of PTFE placed at the bottom of the wells as described above. Other investigators have shown that leukocyte adhesion to PTFE takes place via β_2 integrins [17], although leukocytes also express scavenger receptors that have been shown to act as adhesion molecules on other substrates [22]. To evaluate the respective roles of integrins and scavenger receptors in adhesion to PTFE in the present culture conditions, PBMC were presuspended in DMEM alone or DMEM containing the β_1 integrin ligand-containing peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP, Peninsula, Belmont, CA, USA) at 25 μg/ml for 30 minutes at 37°C before seeding onto polyHEMA-coated tissue culture plates with or without PTFE. Integrin-mediated adhesion is calcium-dependent. Hence, in other experiments PBMC were washed three times with calcium-free PBS and then suspended in calcium-free PBS pH 7.4 containing 5 mM EDTA. Cells were then seeded onto polyHEMA alone or polyHEMA-coated wells containing PTFE. It is plausible that scavenger receptors could play a role in adhesion to PTFE. To determine the role of scavenger receptors in adhesion to PTFE the scavenger receptor ligands polyinosinic acid and dextran sulfate were used as previously described [22]. PBMC were suspended in serum-free DMEM alone or DMEM containing polyinosinic acid at 10, 25 or 50 μg/ml or dextran sulfate at 50, 100 and 1,000 μg/ml. PBMC were preincubated with these agents for

30 minutes at 37°C keeping the cells gently agitated. Peripheral blood mononuclear cell suspensions were then added onto polyHEMA alone or polyHEMA/PTFE wells, and after 60 minutes in all experiments discs were removed from the wells and adherent cells counted using a hemacytometer. For each set of experimental conditions a control sample of cells treated under each of the preincubation conditions and seeded onto polyHEMA coated plates without PTFE was utilized.

To directly visualize leukocyte adhesion to PTFE, which is opaque, J774.16 macrophages (which are larger and more readily visualized than PBMC) were incubated with PTFE fibers in polyHEMA-coated wells. After 60 minutes the fibers were carefully removed, placed on glass slides with a drop of media, overlaid with a coverslip and examined via phase contrast microscopy. This method allows the cells to be easily visualized adhering to and spreading out on the PTFE fibers in the plane perpendicular to the slide surface.

Role of tumor necrosis factor-α in vascular smooth muscle cell proliferation

Tumor necrosis factor-α (TNF-α) is secreted in large quantities by stimulated PBMC and is strongly implicated in mediating VSMC proliferation in a variety of settings including vein grafts, arterial balloon injury and acute cardiac rejection, among others [13–15]. It is thus plausible that enhanced VSMC proliferation in our *in vitro* system could be due to secretion of TNF-α by PBMC. Aliquots of CM from PTFE-incubated PBMC were incubated with neutralizing antibodies to TNF-α (10 μg/ml, Genzyme) or an irrelevant antibody at 10 μg/ml or with no antibody for 60 minutes at 37°C. Aliquots of CM were then diluted in DMEM to achieve a final concentration of 10% and 2% nonheat inactivated FCS. Vascular smooth muscle cells were then incubated with these media and cell proliferation studies carried as described above. In other experiments, VSMC proliferation studies were carried out in media with 2% nonheat inactivated FCS plus varying concentrations of human TNF-α (Sigma).

ELISA measurements for tumor necrosis factor-α

Tumor necrosis factor-α concentrations in supernatants from PBMC incubated on polyHEMA alone or PTFE were measured by using Quantikine human TNF-α Immunoassay Kit (R & D Systems, Inc., Minneapolis, MN, USA). This assay employs a sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TNF-α was precoated onto a microtiter plate. Human TNF-α standards and samples were pipetted into the wells. After washing, an enzyme-linked polyclonal antibody specific for TNF-α was added to the wells. Following a wash a substrate solution was added to the wells and absorbance measured using a Beckman ELISA plate reader. TNF-α concentrations were calculated by comparison with a simultaneously generated standard curve for human TNF-α and were recorded as pg/ml.

Statistical analysis

All data are reported as mean ± SEM and all experiments were carried out in triplicate. Statistical comparisons between two groups were carried out using the Student's unpaired *t*-test. Comparisons between multiple groups were carried out using analysis of variance with Newman-Keuls multiple range testing. Statistical significance was defined as a P value of <0.05.

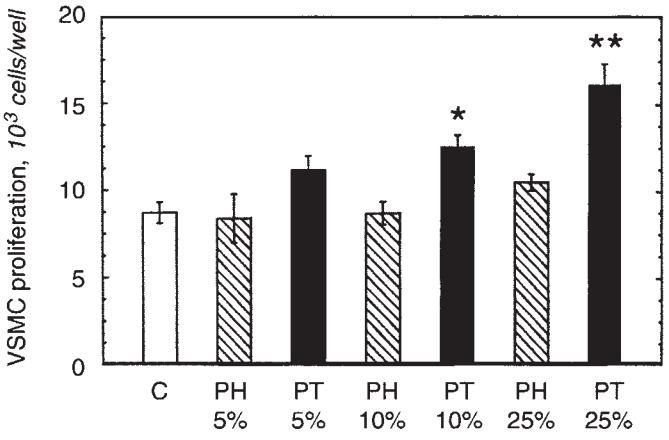

Fig. 1. Vascular smooth muscle cell (VSMC) proliferation following incubation with conditioned media from peripheral blood mononuclear cells (PBMC)/polytetrafluoroethylene (PTFE) interaction. VSMC were seeded onto tissue culture plates, and after six hours the media was changed to DMEM containing 2% nonheat-inactivated FCS with or without conditioned media from PBMC incubated on the nonadhesive substrate polyHEMA alone or on PTFE as described in the **Methods** section. After 72 hours cells were trypsinized and counted using a hemacytometer. Results are from 4 experiments carried out in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. Abbreviations are: C, control, no conditioned media; PH, conditioned media from PBMC in polyHEMA coated wells without PTFE; PT, conditioned media from PBMC in polyHEMA-coated wells containing PTFE. $*P < 0.05$ compared to PH 10%; $**P < 0.01$ compared to PH 25%.

RESULTS

Vascular smooth muscle cell proliferation with PBMC/PTFE conditioned media

Figure 1 illustrates the results of VSMC proliferation studies. A small though nonsignificant increase in VSMC proliferation was observed for VSMC incubated with 5% CM from PBMC that had interacted with PTFE compared to CM from PBMC on polyHEMA alone. However, at 10% concentration of CM a significant increase in VSMC proliferation was observed (10% PBMC/PTFE, 12,500 ± 700 cells; 10% PBMC/polyHEMA, 8,700 ± 650, $P < 0.05$, $N = 4$ experiments carried out in triplicate). A greater increase was seen using 25% CM (25% PBMC/PTFE, 16,130 ± 1,230 cells; PBMC/polyHEMA, 10,500 ± 470, $P < 0.01$, $N = 4$ experiments in triplicate). These data demonstrate that PBMC/PTFE interaction enhances the proliferation of VSMC.

Table 1 summarizes the results of VSMC proliferation studies with PBMC/polyHEMA and PBMC/PTFE CM using pooled uremic sera in place of FCS. Substitution of FCS with uremic sera did not appear to alter the mitogenic effect of PBMC/PTFE on VSMC.

Vascular smooth muscle cell proliferation with conditioned media from J774.16 macrophages incubated with polyetrafluoroethylene

As illustrated in Figure 2, a dose-response relationship can be seen for VSMC incubated with CM from J774.16 incubated with

Table 1. Impact of uremic sera on VSMC proliferation with PBMC/PTFE conditioned media

Condition	Cell number	*P* value
Control	9,200 ± 400	—
5% PBMC/PH CM	10,400 ± 800	NS
5% PBMC/PTFE CM	12,400 ± 400	< 0.05
10% PBMC/PH CM	10,800 ± 500	NS
10% PBMC/PTFE CM	16,800 ± 1,060	< 0.01[a]

Vascular smooth muscle cell (VSMC) proliferation studies were carried out using uremic sera (non-heat inactivated) pooled from 3 patients on chronic hemodialysis in place of fetal calf serum (FCS) with or without conditioned media (CM) as described in the *Methods* section ($N = 4$). Statistical comparisons carried out using analysis of variance with Newman-Keuls multiple range testing. Abbreviations are:

[a] Represents comparison with both control as well as 10% PBMC/PH

PBMC/PH CM, conditioned media collected from PBMC incubated on polyHEMA-coated plates without PTFE; PBMC/PTFE CM, conditioned media collected from PBMC incubated on polyHEMA-coated plates containing discs of PTFE.

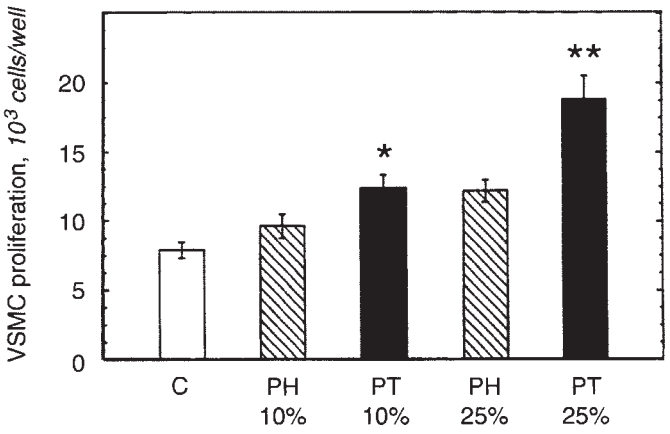

Fig. 2. Vascular smooth muscle cell (VSMC) proliferation studies with conditioned media (CM) from J774.16 macrophages incubated with polytetrafluoroethylene (PTFE). Conditioned media were collected from J774.16 macrophages incubated on polyHEMA alone or polyHEMA-coated plates containing PTFE discs. The VSMC were incubated with media alone or media containing the respective CM and proliferation measured as described in **Methods** section. Data are from 4 experiments carried out in triplicate. Abbreviations are: C, control, no conditioned media; PH, conditioned media from J774.16 macrophages in polyHEMA coated wells without PTFE; PT, conditioned media from J774.16 macrophages in polyHEMA-coated wells containing PTFE. *$P < 0.05$ compared to PH 10%; **$P < 0.01$ compared to PH 25%.

PTFE that is comparable to the findings with CM from PBMC. VSMC proliferation at these concentrations was greater with CM from PTFE-incubated macrophages compared with macrophages on polyHEMA alone. These results with the J774.16 CM are consistent with the PBMC CM findings in that mononuclear cell interaction with PTFE appears to increase VSMC proliferation.

Peripheral blood mononuclear cell adhesion to polytetrafluoroethylene and role of integrins and scavenger receptors

Figure 3A is a representative light photomicrograph of PTFE fibers after incubation with J774.16 macrophages and washing (original magnification ×200). On the side of the fiber perpendicular to the slide surface many of these cells can be seen adhering to and spreading out on the PTFE surface. Figure 3B is a higher magnification view (original magnification ×600) of two of these cells illustrating how they spread out on the PTFE surface. Figure 4 illustrates the results of PBMC adhesion studies. In these experiments 117,300 ± 4,700 PBMC adhered to each PTFE disc. There was no adhesion to polyHEMA-coated surfaces (data not shown). Preincubating PBMC with the β_1 integrin ligand-containing peptide GRGDSP did not significantly attenuate PBMC adhesion to PTFE (115,300 ± 2,900 cells, P = NS, Fig. 4). However, removal of calcium from the incubation media by use of calcium-free media plus 5 mM EDTA significantly diminished adhesion of PBMC to PTFE (22,100 ± 2,100 cells, $P < 0.01$, $N = 3$ experiments carried out in triplicate, Fig. 4). These findings are consistent with those of other investigators in that they suggest that β_2 integrins mediate adhesion of PBMC to PTFE [17].

While scavenger receptors play an important role in leukocyte adhesion to other substrates such as plastic, it is unknown whether scavenger receptors could play any significant role in PBMC adhesion to PTFE. As shown in Figure 5 preincubating PBMC with the scavenger receptor ligands dextran sulfate and polyinosinic acid, even at high concentrations, did not attenuate PBMC adhesion to PTFE. These data demonstrate that scavenger receptor interaction seems unlikely to play any significant role in PBMC adhesion to PTFE.

Effect of neutralizing antibodies to tumor necrosis factor-α on vascular smooth muscle cell proliferation

Figure 6 illustrates the results of VSMC proliferation studies carried out using CM preincubated with neutralizing antibodies to TNF-α. As this Figure illustrates, the mitogenic effect of PBMC/PTFE CM was significantly attenuated by neutralizing antibodies to TNF-α (control, 8,500 ± 280 cells; 10% PBMC/PTFE CM, 12,540 cells, $P < 0.05$; 10% PBMC/PTFE/anti-TNF-α, 9,500 ± 640 cells, $P < 0.05$, $N = 4$ experiments carried out in triplicate). The mitogenic effect of PBMC/PTFE CM was not attenuated by preincubation with an irrelevant antibody (data not shown).

Tumor necrosis factor-α production following peripheral blood mononuclear cell interaction with polytetrafluoroethylene

Peripheral blood mononuclear cell interaction with PTFE led to a marked increase in TNF-α levels in the CM compared to PBMC incubated with polyHEMA alone (control PBMC [polyHEMA], 1.3 ± 0.3 pg/ml; PBMC/PTFE, 1,150 ± 100 pg/ml, $P < 0.01$, $N = 3$). These data, which are illustrated in Figure 7, demonstrate that PBMC generation of TNF-α is greatly enhanced by interaction with PTFE compared to being kept in suspension.

Direct effect of human tumor necrosis factor-α on vascular smooth muscle cell proliferation

As summarized in Table 2, human TNF-α exerted a concentration-dependent effect on VSMC proliferation. The earliest statistically significant effect on proliferation could be detected at incubation with a concentration of 10 pg/ml. Based on the concentrations of TNF-α detected by ELISA and these data, the dilutions of CM used in the present experiments contained concentrations of TNF-α correlating with enhanced VSMC proliferation.

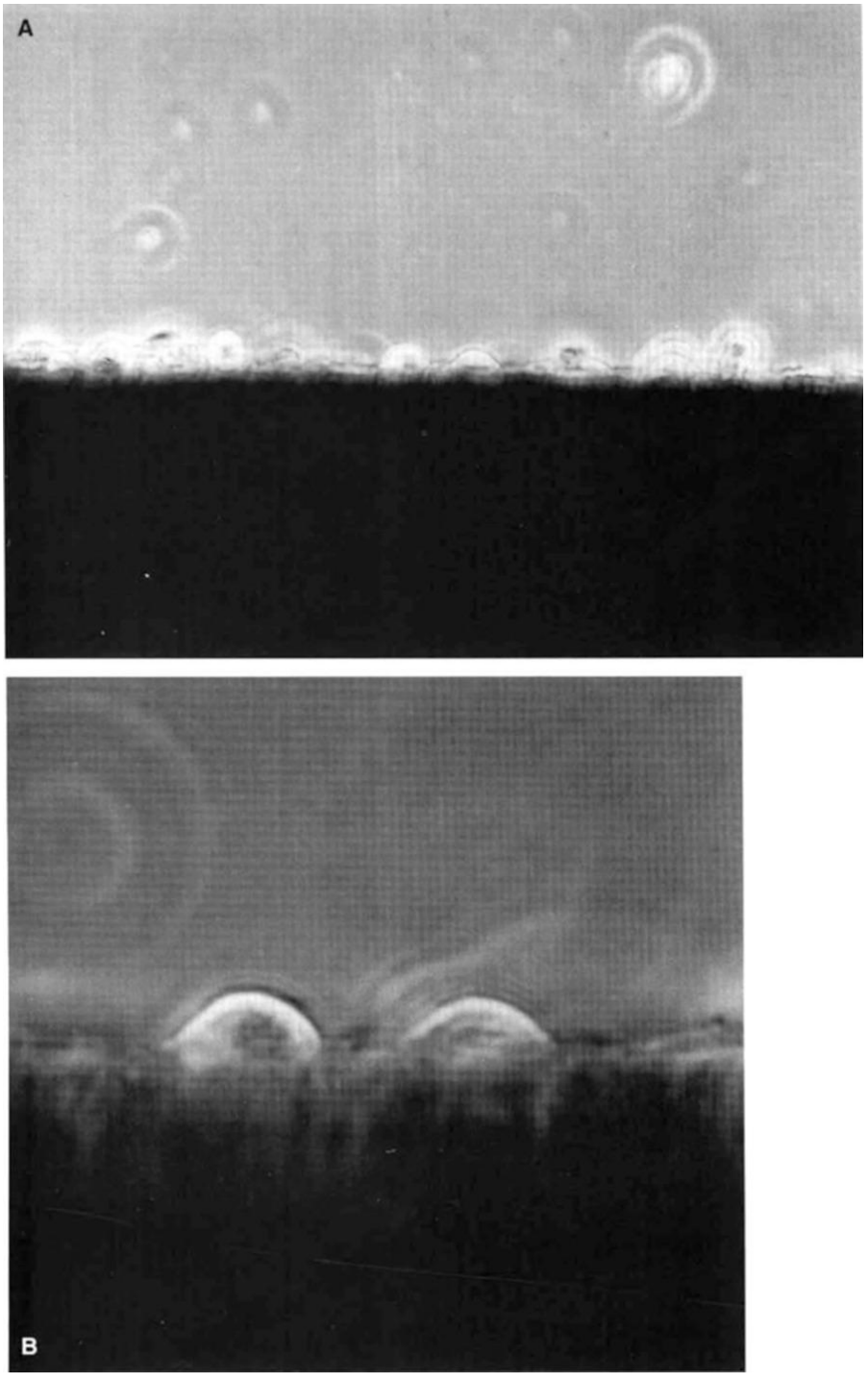

Fig. 3. Representative light photomicrographs of J774.16 macrophages adherent to polytetrafluoroethylene (PTFE). J774.16 macrophages (used because of their larger size and hence easier visualization) were incubated with PTFE fibers in polyHEMA coated wells for 60 minutes at 37°C, removed from the wells, placed on glass microscope slides with a drop of media, overlaid with a coverslip and examined using phase contrast microscopy. (*A*) Many cells can be seen adhering and spreading out over the perpendicular surface of the PTFE material (original magnification, ×200). (*B*) A higher magnification view (original magnification, ×600) of two of the cells adhering and spreading on the PTFE material.

DISCUSSION

One of the most troubling problems in hemodialysis patients is maintaining long-term functioning angioaccess, particularly in those who have arteriovenous PTFE grafts. In many patients, though, this is the preferred form of angioaccess because of poor vasculature and other conditions [23]. In the United States the placement of arteriovenous grafts made of PTFE far exceeds the creation of arteriovenous fistulas and vascular access-related problems account for at least 15% of hospital admissions in these patients. While some studies have suggested a role for hypercoagulable states in access thrombosis and while undoubtedly this does contribute to access failure in some patients, it has been shown that the most common reason for failure of arteriovenous grafts is the development of intimal hyperplasia at the venous anastomosis of the grafts [5, 12]. The hyperplastic cells in the intimal hyperplastic lesion have been shown to be VSMC [12, 24].

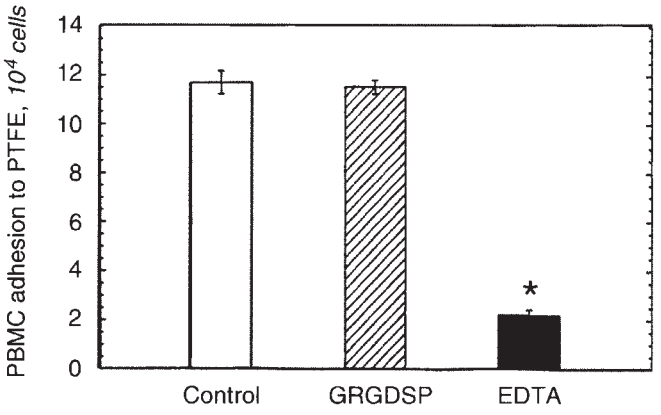

Fig. 4. Role of integrins in peripheral blood mononuclear cell (PBMC) adhesion to polytetrafluoroethylene (PTFE). The PBMC were preincubated with an integrin ligand-containing peptide GRGDSP at 25 μg/ml for 30 minutes as described in the **Methods** section. Other aliquots of PBMC were washed in calcium and magnesium-free PBS and were then suspended in calcium and magnesium-free PBS containing 5 mM EDTA. The PBMC were then seeded onto polyHEMA coated wells containing PTFE discs and adhesion studies carried out as described in the **Methods** section. Data are from 3 experiments carried out in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. $^*P < 0.01$ compared to control.

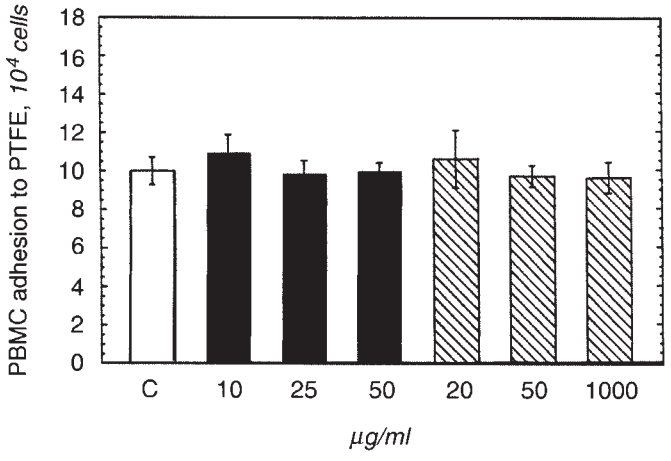

Fig. 5. Role of scavenger receptors in peripheral blood mononuclear cell (PBMC) adhesion to polytetrafluoroethylene (PTFE). The PBMC were presuspended in media alone (□) or media containing either polyinosinic acid at 10, 25 and 50 μg/ml (■) or dextran sulfate at 20, 50, 100 and 1,000 μg/ml (▨). After a 30 minute preincubation, PBMC were seeded onto wells coated with polyHEMA or polyHEMA-coated wells containing PTFE discs. After 60 minutes, adhesion studies were carried out as described in the **Methods** section. Data are from 3 experiments carried out in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. There were no statistically significant differences in adhesion between any of the experimental conditions.

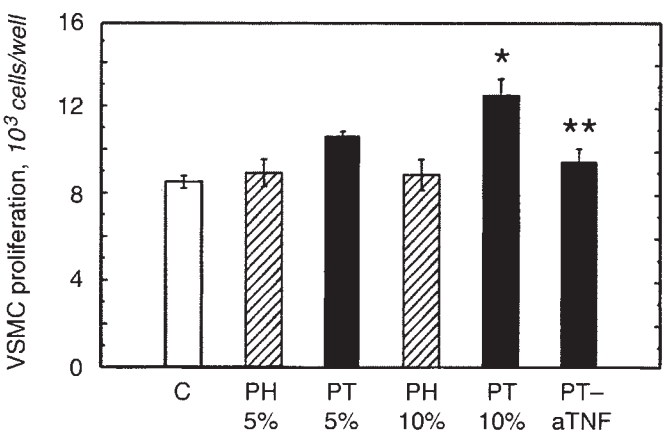

Fig. 6. Role of tumor necrosis factor-alpha (TNF-α) in modulation of vascular smooth muscle cell (VSMC) growth by peripheral blood mononuclear cell (PBMC)/polytetrafluoroethylene (PTFE) interaction. Conditioned media (CM) were collected from PBMC that were incubated with PTFE as described in the **Methods** section. Aliquots were then incubated with or without neutralizing antibodies to TNF-α (10 μg/ml) or an irrelevant antibody (10 μg/ml) for 60 minutes at 37°C. VSMC were then incubated with DMEM containing 2% nonheat-inactivated FCS with or without 10% CM from the above experimental conditions and VSMC proliferation measured as described in the **Methods** section. Data are from 4 experiments carried out in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. Abbreviations are: C, control, no conditioned media; PH, conditioned media from PBMC in polyHEMA coated wells without PTFE discs; PT, conditioned media from PBMC in polyHEMA-coated wells containing PTFE discs; PT-aTNF, conditioned media from PBMC in polyHEMA-coated wells containing PTFE discs that were incubated with neutralizing antibodies to TNF-α prior to VSMC proliferation studies. $^*P < 0.05$ compared to PH 10%; $^{**}P < 0.05$ compared to PT 10%.

However, no satisfactory hypothesis exists to account for why arteriovenous grafts fail at a higher rate compared to fistulas. While some studies have shown that certain clinical variables correlate with early angioaccess failure [9, 10], these studies have not addressed mechanisms for the disproportionate failure of arteriovenous grafts. Unfortunately, to date therapeutic maneuvers such as systemic anticoagulation have been unsuccessful in prolonging arteriovenous graft function, except perhaps in those patients with hypercoagulable states such as that associated with the antiphospholipid antibody syndrome. For the majority of hemodialysis patients such therapies are ineffective.

Tumor necrosis factor-α is a well-known VSMC mitogen that has been demonstrated to play a role in VSMC proliferation in a variety of vascular pathologies [13–15] and PBMC are a rich source of this cytokine. The present study demonstrates that PBMC adhesion to PTFE markedly enhances the generation of TNF-α compared to nonadherent PBMC, and promotes the proliferation of VSMC. We were able to attenuate the mitogenic effect of the conditioned media using neutralizing antibodies to TNF-α and found a marked increase in TNF-α secretion by PBMC that had interacted with the PTFE material using ELISA. Hence, this study suggests a potential mechanism by which intimal hyperplasia at the venous anastomosis of arteriovenous grafts may develop, namely, circulating leukocytes may adhere to this graft material and become activated to secrete TNF-α and perhaps other cytokines that get carried downstream where they can bind to receptors on VSMC at the venous anastomosis and increase their proliferation. However, it is likely that, if this indeed occurred *in vivo*, it would best be regarded as one of several variables contributing to the development of this lesion along with turbulence, platelet adhesion at needle puncture sites and other factors with suspected roles in this disorder.

Prior reports have not excluded a potential role for scavenger receptor interaction in this process. Leukocytes express scavenger

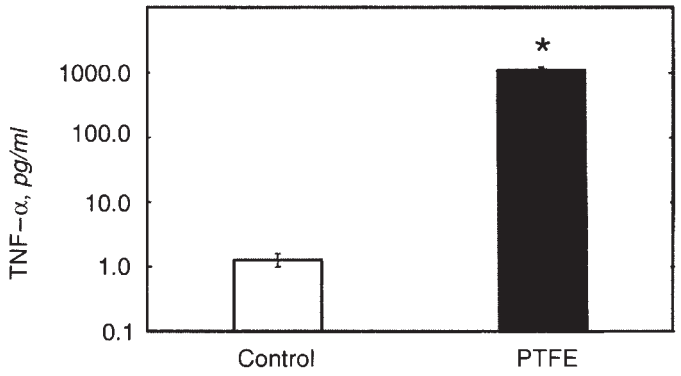

Fig. 7. Tumor necrosis factor alpha (TNF-α) production by peripheral blood mononuclear cells (PBMC) exposed to polytetrafluoroethylene (PTFE). The PBMC were seeded onto wells coated with polyHEMA (control) or wells coated with polyHEMA containing a disc of PTFE (PTFE in figure) as described in the **Methods** section. After a 24 hour incubation at 37°C, TNF-α levels in the culture supernatants were measured using a sandwich enzyme immunoassay technique as described in the **Methods** section. Data are from 3 experiments in triplicate and are expressed as mean ± SEM pg TNF-α per ml PBMC culture supernatant. Note that the y axis (TNF-α level) is in a logarithmic format. Statistical comparison carried out using the Student's unpaired *t*-test. Control: PBMC on wells coated with polyHEMA without PTFE; PTFE: PBMC on polyHEMA coated wells containing PTFE discs. $^*P < 0.01$.

Table 2. Direct effect of human TNF-α on VSMC proliferation

Concentration *pg/ml*	Cell number	*P* value
Control	10,000 ± 1,100	—
0.1	11,700 ± 480	NS
1.0	15,200 ± 1,200	NS
10	17,500 ± 3,010	< 0.05
100	19,200 ± 750	< 0.05
1000	21,300 ± 3,180	< 0.01

Vascular smooth muscle cells (VSMC) were seeded onto 96-well plates and incubated in 2% nonheat inactivated fetal calf serum (FCS) with varying concentrations of human tumor necrosis factor alpha (TNF-α) and cell counts determined as described in the **Methods** section ($N = 3$). Statistical analysis was carried out using analysis of variance with Newman-Keuls multiple range testing. *P* values correspond to comparison with control.

receptors, and while they appear to play a role mostly in taking up modified substances such as oxidized LDL, they can also act as adhesion molecules, as it has been shown, for example, that leukocyte adhesion to another synthetic surface (plastic) is scavenger receptor mediated [25], as is adhesion to oxidized extracellular matrix [22]. While we found significant adhesion of PBMC to PTFE, we could not antagonize this adhesion using the scavenger receptor ligands polyinosinic acid and dextran sulfate, even at very high concentrations. Use of the soluble β_1 integrin ligand GRGDSP did not significantly attenuate adhesion to PTFE while calcium-free media with EDTA, however, which will inhibit binding by both β_1 and β_2 integrins to a ligand-containing substrate, did attenuate adhesion. Our findings appear to exclude any significant contribution of scavenger receptors in leukocyte adhesion to PTFE and suggest that in our system it was predominantly β_2 integrin-mediated, consistent with findings of other investigators. Our findings are also consistent with the observation that integrin binding to ligand enhances TNF-α production [26], and based on our VSMC proliferation studies these findings suggest that this may have important implications for the development of venous intimal hyperplasia. Over time the TNF-α release by leukocytes activated via interaction with PTFE may expose VSMC at the venous anastomosis to this mitogen, resulting in the development of the lesion described by Swedberg et al in their pioneering study as suggestive of "... a steadily progressive (rather than episodic) smooth muscle hyperplasia..." [12].

While PTFE does not endothelialize in humans and in our experimental protocol we evaluated adhesion directly to the graft material, *in vivo* this substrate may be coated with a variety of plasma proteins and hence only part of the PTFE surface may be exposed. Alternatively, the major stimulus for PBMC activation may take place in the early postoperative period, with leukocytes adhering extensively to the uncoated surface, causing an initial strong stimulus for VSMC proliferation with this stimulus subsequently diminishing yet continuing.

If leukocyte activation and release of VSMC mitogens such as TNF-α as a consequence of adhesion to PTFE plays a significant role in the development of venous intimal hyperplasia, then one can envision a number of approaches that theoretically might be able to disrupt this sequence of events. The most obvious of these would consist of somehow modifying the luminal surface of the PTFE graft to prevent integrin-mediated adhesion and cross-linking. Indeed, some investigators have shown that modifications of the PTFE surface can decrease adhesion and can cause some decrease in TNF-α production [27], though these studies did not report TNF-α production by nonadherent cells (by contrast, we found a large increase in TNF-α production by PBMC on PTFE compared to those in suspension). While this may appear to be a logical approach, a surface incapable of allowing integrin-mediated adhesion might pose a risk of serious hemorrhage following removal of hemodialysis needles from the graft, as platelet aggregation at sites of injury (needle puncture) is integrin-dependent. Without any change in the nature of the graft material itself, an alternative approach might be to modulate the leukocyte response to the PTFE surface. This might be done by using soluble β_2 integrin ligands or agents that could prevent the cross linking of integrins. Alternatively, one or more of the subsequent events after integrin cross linking might be antagonized, such as tyrosine phosphorylation of some of the proteins comprising focal adhesion complexes at these sites. Agents that could specifically inhibit production of TNF-α and other cytokines or the VSMC response to mitogenic stimuli (perhaps by directly modifying the VSMC cells at the venous anastomosis by transfection with genes promoting quiescence) would be theoretical possibilities as well. These highly speculative approaches would first require *in vivo* demonstration of leukocyte generation of TNF-α in the pathogenesis of venous intimal hyperplasia in arteriovenous grafts.

In summary, the present study demonstrates that PBMC interaction with PTFE promotes the proliferation of VSMC at least in part due to increased TNF-α generation. These data support the hypothesis that arteriovenous graft failure from venous intimal hyperplasia may be a consequence of leukocyte interaction with PTFE graft material. Further understanding of this mechanism might yield more effective medical therapies to prevent angioaccess failure in hemodialysis patients with arteriovenous grafts.

ACKNOWLEDGMENTS

This work was supported by a Young Investigator Award from the National Kidney Foundation of New York/New Jersey (J.M.), and by a National Institutes of Health grant 2RO1 DA 06753 (P.C.S.).

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APPENDIX

Abbreviations used in this article are: VSMC, vascular smooth muscle cells; PBMC, peripheral blood mononuclear cells; PTFE, polytetrafluoroethylene; TNF-α, tumor necrosis factor-alpha; CM, conditioned media; polyHEMA, polyhydroxyethylmethacrylate; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's MEM; FCS, fetal calf serum.

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