Basic Fibroblast Growth Factor (bFGF, FGF-2) Potentiates Leukocyte Recruitment to Inflammation by Enhancing Endothelial Adhesion Molecule Expression

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Basic fibroblast growth factor (bFGF, FGF-2) is a potent angiogenic factor and endothelial cell mitogen. Although bFGF levels are increased in chronically inflamed tissue, its role in inflammation is unclear. We investigated the effect of bFGF on acute dermal inflammation and the recruitment of monocytes, T cells, and neutrophils. Leukocyte recruitment to inflamed sites was quantified with radiolabeled leukocytes. Intradermal injection of bFGF in rats did not induce leukocyte recruitment or inflammation. However, the recruitment of leukocytes to inflammation induced by tumor necrosis factor- α , interferon- γ , C5a, or a delayed hypersensitivity reaction was enhanced by bFGF by 55 to 132% ($P < 0.05$). Either acute or prolonged bFGF treatment of dermal sites had this effect. The potentiating effect of bFGF on leukocyte recruitment was also seen in joints. There was no associated modulation of vascular permeability, blood flow, or angiogenesis in the sites by bFGF. However, the expression of the endothelial cell adhesion molecules (CAMs) for leukocytes, P-selectin, E-selectin, and ICAM-1, was significantly up-regulated in the inflamed tissue by bFGF, as quantified by radiolabeled anti-CAM antibody binding *in vivo*. Thus, although not directly proinflammatory, bFGF synergistically potentiates inflammatory mediator-induced leukocyte recruitment, at least in part, by enhancing CAM up-regulation on endothelium. (Am J Pathol 2006; 168:835–846; DOI: 10.2353/ajpath.2006.050479)

The interaction of leukocytes with vascular endothelial cells (ECs) and their emigration from the blood by trans-endothelial migration are essential components of the

inflammatory reaction, defense against infections, and development of immunity. This process involves a cascade of events in which engagement between cell adhesion molecules (CAMs) on the surface of activated ECs and on the surface of leukocytes occurs. Leukocyte capture by and rolling on ECs are mediated by endothelial E- and P-selectin and $\alpha 4$ integrins while firm adhesion and migration of rolling leukocytes is primarily dependent on $\beta 2$ integrins on leukocytes binding to intercellular CAM-1 (ICAM-1) and ICAM-2 on ECs.¹ The activation of ECs for expression of CAMs is mediated in part by inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). Chemotactic factors, such as interleukin (IL)-8, C5a, or MCP-1, are also involved in the recruitment of leukocytes to sites of inflammation.²

The inflammatory process is often accompanied by angiogenesis (formation of new blood vessels from pre-existing vasculature) and this is essential for tissue repair. Tumor growth is also dependent on angiogenesis. A variety of angiogenic factors are known to induce EC migration and proliferation. Basic fibroblast growth factor (bFGF, FGF-2) has been associated with tumor angiogenesis^{3,4} and tissue healing.^{5–7} bFGF binds to cell-surface tyrosine kinase receptors (FGFR), an interaction that is modulated by the presence of heparin-heparan sulfate⁸ and that increases the stability of the FGF-FGFR complex and its efficacy of signaling.⁹

The role of CAMs in leukocyte adhesion and emigration has been studied in normal microvessels. However, the mechanisms of leukocyte interaction with ECs in angiogenic vessels are poorly understood. Angiogenic vessels may have significantly altered interactions with blood

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leukocytes.^{10–12} For example, in tumor neovessels leukocyte interactions with the ECs are diminished.^{13–17} These findings have been related to diminished expression of EC-CAMs such as ICAM-1, ICAM-2, VCAM-1, and E-selectin in tumor vessels,^{18–20} perhaps due to inhibition of expression by bFGF and another angiogenic factor found in tumors, vascular endothelial growth factor.^{10,21} Recently, our laboratory demonstrated *in vitro* that long-term (ie, more than 24 hours) treatment of human umbilical vascular endothelial cells (HUVECs) with bFGF down-modulated the adhesion and transendothelial migration of monocytes and neutrophils (PMNs). Furthermore, endothelial expression of adhesion molecules and chemokines was also inhibited.^{22,23}

It is well known that expression and secretion of angiogenic factors including bFGF is increased at sites of chronic inflammation. bFGF is increased in serum and affected tissue of patients with rheumatoid arthritis, inflammatory bowel disease, or asthma.^{24–27} However, it is not known whether the putative effect of such factors on EC and leukocyte recruitment is comparable in tumors and in inflammation. During inflammation, the presence of angiogenic factors has been associated with more intense accumulation of leukocytes and exacerbation of injury.^{28–30}

The aim of this work was to elucidate the effects of bFGF on the recruitment of leukocytes during inflammation in normal tissue using skin as a model. Here, we provide *in vivo* evidence that bFGF synergistically enhances the recruitment of monocytes, T cells, and PMNs in response to a variety of inflammatory mediators. We also demonstrate that one of the mechanisms involved in the bFGF effect is increased expression of adhesion molecules on cytokine-activated ECs.

Materials and Methods

Animals

Inbred male Lewis rats (Charles River Canada, St. Constant, QC, Canada), weighing 250 to 300 g, were used. Some animals were used exclusively for harvesting of blood leukocytes for purification as previously described,³¹ whereas others were used for dermal inflammatory reactions and were recipients of labeled leukocytes. Animals in which a delayed type hypersensitivity (DTH) reaction was to be induced were immunized with 0.05 mg of killed *Mycobacterium butyricum* (Difco Laboratories, Inc., Detroit, MI) in 0.05 ml of mineral oil subcutaneously in two sites at the base of the tail.

Reagents

Human bFGF, rat TNF- α , rat IFN- γ , and rat MIP-2 were purchased from Peprotech Inc. (Rocky Hill, NJ). Recombinant rat bFGF was a kind gift from E. Kardami and P. Cattini (the Department of Physiology and the Department of Anatomy, respectively, University of Manitoba, Winnipeg, MB, Canada). Heparin (molecular weight

~3000 d, from porcine intestinal mucosa) was from Sigma Chemical Co. (St. Louis, MO). Zymosan-activated serum (ZAS) was generated by activating complement in normal rat serum (37°C, 60 minutes) with Zymosan A (Sigma), followed by removal of the zymosan by centrifugation as previously described.³¹ This ZAS was used as a source of the chemotactic factor C5a_{des-arg}. Fetal calf serum (MediCorp, Montreal, QC, Canada) contained <0.0315 ng/ml of endotoxin. Human serum albumin (HSA), endotoxin- and pyrogen-free, was obtained from Connaught Laboratories (Downsview, ON, Canada). RPMI 1640 (Sigma) and all salts for buffers [Tyrode's solution, phosphate-buffered saline (PBS)] were made with analytical grade chemicals and dissolved in endotoxin- and pyrogen-free water (Abbott Laboratories, Ltd., St.-Laurent, QC, Canada). The following anti-CAM mouse IgG mAbs were used: RMP-1 and RP-2 (to rat P-selectin);³² RME-1 and 14G2 (to rat E-selectin);³³ 1A29, WT-3, and TA-2 (to rat ICAM-1, rat CD18, and rat VLA-4, respectively; kind gifts from M. Miyasaka, Osaka, Japan and T.B. Issekutz, Halifax, NS, Canada);^{34–36} and 5F10 (to VCAM-1; a kind gift from L. Burkly, Biogen Inc., Cambridge, MA).³⁷

Rat Leukocyte Purification and Labeling

Rat blood PMNs and monocytes for migration studies were isolated by hydroxyethyl starch exchange transfusion technique as described previously.³¹ Briefly, via the femoral vein of an anesthetized heparinized donor rat, blood was gradually exchanged with 6% hydroxyethyl starch/saline (Hespan; American Hospital Supply Corp., McGaw Park, IL) into acid-citrate-dextrose (ACD, formula A; Fenwal-Travenol, Malton, ON, Canada) anti-coagulant, and red blood cells were sedimented at $1 \times g$. The leukocyte-rich plasma was harvested, and the leukocytes were pelleted ($200 \times g$ for 10 minutes) and resuspended in calcium- and magnesium-free Tyrode's solution (TyS^{−/−}) containing 10% platelet-poor plasma (PPP). Leukocytes were layered on discontinuous Percoll gradients (63%/74%). Cells recovered at the interphase between 63% and 74% Percoll were >95% neutrophils. The PMNs were washed and labeled with ¹¹¹In-labeled oxine (Amersham Corp., Oakville, ON, Canada) at $1.5 \mu\text{Ci}/1 \times 10^7$ PMNs in 0.1 ml of TyS^{−/−} solution for 10 minutes at room temperature. Labeled PMNs were washed three times with and resuspended in TyS^{−/−}-10% PPP for intravenous injection.

Monocytes were purified as described,³¹ with minor modifications. Briefly, the mononuclear layer on the top of 63% Percoll was removed, washed, and resuspended in 3.6 ml of TyS^{−/−}-10% PPP, and the osmolality of this suspension was slightly increased by the addition of 20 μl of 9% NaCl to improve the separation of monocytes from lymphocytes. After incubation for 10 minutes at 37°C, this cell suspension was layered onto another discontinuous Percoll gradient composed of 40% Percoll, on top of 55% Percoll, on top of 59% Percoll. All of the Percoll layers had 12.5 μl of 9% NaCl/2.5 ml of gradient. The gradient was centrifuged

at $400 \times g$ for 30 minutes at room temperature, and monocytes were harvested at the 40%/55% and the 55%/59% interphases. The purified monocytes (>85%) were washed and radiolabeled with 75 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (Amersham) per 5×10^7 monocytes in 1 ml of TyS^{-/-}-10% PPP for 30 minutes at 37°C. Labeled monocytes were washed twice and resuspended in TyS^{-/-}-10% PPP for intravenous injection.

T lymphocytes were purified from rat spleen as previously described³⁸ with minor modifications. Briefly, the spleen was minced in RPMI 1640 to release the cells. The cells were washed, resuspended in RPMI-10% fetal calf serum, and allowed to penetrate a sterile, pyrogen-free nylon wool column. The column was clamped and incubated 1 hour at 37°C. The purified T cells (>90%) were eluted with RPMI-fetal calf serum, centrifuged, and labeled with ^{111}In as described for PMNs. Each rat received 5×10^6 ^{51}Cr -labeled monocytes together with 10×10^6 ^{111}In -labeled PMNs, or 20×10^6 ^{111}In -labeled T cells.

Vascular Permeability, Vascular Density, and Blood Flow Measurements

Exudation due to enhanced vascular permeability was quantitated using HSA labeled with ^{125}I , as described.³⁹ An aliquot of ^{125}I -HSA (5 $\mu\text{Ci/kg}$) was administered intravenously 2 hours before sacrifice. The content of ^{125}I per site and of ^{125}I per μl of blood plasma was quantified, and content in skin sites expressed as μl of plasma albumin equivalents/site.

Vascular density was measured by blood volume in the skin sites by determining the content of the intravascular marker ^{125}I -IgG. This is a modification of the method using fluorescein isothiocyanate-labeled dextran (molecular weight ~ 150 kd).⁴⁰ A 5- to 10- $\mu\text{Ci/kg}$ aliquot of ^{125}I -IgG was injected intravenously into each rat 15 minutes before sacrifice. The content of ^{125}I per site was quantified and expressed as the fold increase over the control sites. Blood flow was measured as previously described⁴¹ with minor modifications. Briefly, just before sacrifice, 50 μCi of $^{86}\text{RbCl}$ (Amersham) were injected intravenously. Forty-five seconds later, 1 ml of saturated KCl solution was injected intravenously to cause cardiac arrest. The content of ^{86}Rb in the skin sites was quantified and expressed as fold increase over the control sites.

Growth Factor Pretreatments and Inflammatory Reactions

The dorsal skin of Lewis rats was shaved and dermal sites were injected intradermally in triplicate with 50 μl of 2, 10, 50, 100, or 200 ng of bFGF and 5 μg of heparin or heparin alone (control) in RPMI-HSA (1 mg/ml). Because bFGF and heparin did not induce inflammation, neither was considered an inflammatory stimulus. These injections were made at various stages: 48 hours before and at the time of injection of inflammatory stimuli (days -2

and 0), daily for 5 days (days -5 to 0), only 48 hours before (day -2), or only at the time of inflammatory stimulus (day 0). Inflammatory reactions were induced by intradermal injection (50 μl) of various inflammatory stimuli in triplicate sites in the dorsal skin in the bFGF and heparin or heparin (control)-pretreated sites. These included 3 ng of TNF- α , 100 U of IFN- γ , 3 ng of TNF- α and 100 U of IFN- γ , 10% ZAS as source of C5a_{des-Arg}, 10 μg of killed *M. butyricum* and 20 ng of MIP-2, all in RPMI-HSA (0.1%). Inflammatory agents were injected at the time of intravenous injection of radiolabeled leukocytes. The animals were sacrificed 18 hours or 2 hours later as indicated.

In some experiments, the recruitment of leukocytes to inflamed sites was blocked by intravenous injection of anti-rat CD18 and anti-rat CD49d antibodies (WT-3, 1 mg/rat and TA-2, 0.5 mg/rat) immediately before induction of inflammation and injection of radiolabeled leukocytes. The absence of leukocyte recruitment after 2 hours was verified as reported previously.^{38,42,43} Where the effect of rat bFGF was compared with its human equivalent, dermal sites were injected intradermally in triplicate with 50 μl of 50 ng rat or human recombinant bFGF and 5 μg of heparin $\pm$ 3 ng of TNF- α and 100 U of IFN- γ . ^{111}In -labeled PMNs were injected intravenously. The animals were sacrificed 2 hours later.

In some experiments, PMN recruitment to joints was investigated. Inflammatory reactions were induced in the ankles and wrists by injection in 20 μl of 100 ng of TNF- α and 300 U of IFN- γ with 5 μg of heparin $\pm$ 50 ng of human bFGF or rat bFGF in diluent (RPMI-HSA 1 mg/ml). Immediately thereafter, ^{111}In -labeled PMNs (10×10^6) were injected intravenously. The animals were sacrificed 2 hours later.

Measurement of Leukocyte Accumulation

At the time of sacrifice, 2 ml of blood were collected in acid-citrate-dextrose anti-coagulant, and dorsal skin was removed and cleaned. The inflamed sites were punched out (12-mm diameter punch) and the joints were removed. Samples of spleen, liver, lung, and lymph nodes were taken for determination of ^{111}In and ^{51}Cr content. Counts of ^{111}In and ^{51}Cr in the tissues were expressed as cpm detected per 10^6 cpm injected. The amount of radioactivity (^{111}In , ^{51}Cr , ^{125}I , ^{86}Rb) was measured in a four-channel gamma spectrometer (LKB1282; Fisher Scientific Co., Ottawa, ON, Canada). Automatic corrections were made for the spill of isotope emissions into adjacent channels.

Histology in Dermal Inflammatory Sites

The dermal lesions were transected and fixed in 2.5% acetic acid, 2% formalin, and 75% ethanol (AFA fixative) for 24 hours at room temperature. Then, 1-mm slices were placed in 70% ethanol until paraffin embedding. Sections (5 μm thick) were cut, deparaffinized, and rehydrated by sequential passage in xylol and 100%, 95%, and 70% ethanol. The endogenous peroxidase was

quenched with 3% H₂O₂-methanol 1:1 (30 minutes), and nonspecific binding of antibodies was blocked with Tris-buffered saline, 1% bovine serum albumin (Sigma), 10% normal goat serum, egg white avidin (1 µg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 0.02% Tween 20 (Sigma) (20 minutes, 22°C). The slides were incubated with rabbit anti-mouse laminin antibody (Cedarlane Laboratories Ltd., Hornby, ON, Canada) or nonimmune rabbit IgG (Jackson ImmunoResearch Laboratories) overnight at 4°C. The secondary antibody was a biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The assay was developed with ABC-DAB kit (Vector Laboratories, Burlingame, CA) as suggested by the manufacturer. The slides were dehydrated and mounted in Permount (Fisher Scientific).

To visualize CAM expression, the dermal lesions were fixed in Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) for 24 hours and then transferred to 80% ethanol until paraffin embedding. Sections (5 µm) were deparaffinized and rehydrated, and nonspecific binding of antibodies was blocked as above. The slides were incubated with primary mAb (eg, to rat E-selectin, to rat P-selectin, or IgG1 isotype control) overnight at 4°C. The secondary antibody, a biotin-conjugated goat anti-mouse IgG (Sigma), was incubated 1 hour at room temperature, and the binding was detected after washing with streptavidin-Alexa Fluor 568 (2 µg/ml, incubation 60 minutes; Molecular Probes, Inc., Eugene, OR) followed by washing, slide mounting, and examination with an immunofluorescence microscope.

For ICAM-1 visualization, skin sites were snap-frozen in liquid nitrogen-cooled isopentane and embedded in OCT (Tissue-Tek; Sakura Finetek U.S.A., Inc., Torrance, CA). Cryostat sections (8 µm) were air-dried and acetone-fixed for 5 minutes. After treatment with normal goat serum, the sections were incubated 1 hour with mouse anti-rat ICAM-1 mAb (1A29) or isotype control (10 µg/ml). The subsequent steps were performed under the same conditions as the ones used for E- and P-selectin staining.

Vessel Density Quantification

The skin sections were examined in a Nikon Eclipse E600 microscope (Nikon Canada Inc., Mississauga, ON, Canada) and analyzed with ACT-1 program in digital images. The number of laminin-stained vessels in a 0.8-mm² area was counted, differentiating between small, medium, or large vessels. Vessels were considered small when their lumens were ~1 leukocyte in diameter, medium when 2 to 3 in diameter and large when more than 3. Only vessels with defined borders and visualized in cross-section were counted.

Radiolabeling of mAb

The *in vivo* expression on ECs of E-selectin, P-selectin, VCAM-1, and ICAM-1 was quantified by the binding in the skin sites of intravenously administered ¹²⁵I- or ¹³¹I-labeled antibodies as previously described.⁴⁴ Briefly,

100 µg of mAb were incubated with 100 µCi of ¹²⁵I or ¹³¹I in an Iodogen-precoated tube (Pierce Biotechnology, Inc., Rockford, IL) for 15 minutes at room temperature. The reaction was stopped by removing the sample from the tube. The free isotope was separated by dialyzing overnight against PBS and then diluting and reconcentrating the sample in a centrifugal filter device with a molecular-weight cutoff of 30,000 d (30,000 NMWL; Millipore Corp., Bedford, MA).

Quantification of Vascular Adhesion Molecule Expression with Radiolabeled mAb

Endothelial expression of E-selectin, P-selectin, VCAM-1, and ICAM-1 in inflamed dermal sites was determined by an intravenous injection of ¹²⁵I-labeled RME-1 (anti-rat E-selectin), RMP-1 (anti-rat P-selectin), 1A29 (anti-rat ICAM-1), or 5F10 (anti-rat VCAM-1) mAb plus ¹³¹I-labeled control IgG (mAb 11D10 anti-human neutrophil, unpublished). Briefly, the animals were anesthetized with Ketamine-Innovar (SABEX Inc., Quebec, QC, Canada) subcutaneously, and 10 minutes later a mixture of 25 µg of ¹²⁵I anti-CAM mAb plus 25 µg of control IgG was injected intravenously. Immediately thereafter, the animals received an intraperitoneal injection of 0.4 ml of xylazine. After 4 minutes the abdomen was opened, the ascending aorta cannulated, and 1 ml of 200 U heparin in saline was administered. The inferior vena cava was then opened, and the animals were perfused via the aorta with 75 ml of TyS^{+/+} buffer. The content of ¹²⁵I and ¹³¹I in skin sites was quantified in a gamma counter along with a known fraction of the total injected dose of each labeled antibody. The expression of CAMs in test and control injected skin sites was calculated as the percentage of the ¹²⁵I-injected dose (% ID) – the percentage of ¹³¹I ID of isotype control IgG – normal (noninjected) skin.

Statistical Analysis

Statistical significance was determined using paired *t*-test. *P* values exceeding 0.05 were considered not significant.

Results

Leukocyte Recruitment in Response to Inflammatory Mediators Is Up-Regulated by bFGF

Previously, we demonstrated *in vitro* that the pretreatment of ECs with bFGF for 1 and up to 3 days down-modulates EC activation for the expression of adhesion molecules and production of some chemokines, associated with a decrease in transendothelial migration of PMNs and monocytes.^{22,23} To study this action of bFGF *in vivo*, the migration of ⁵¹Cr-labeled blood monocytes and ¹¹¹In-labeled spleen T cells or ¹¹¹In-labeled PMNs to various inflammatory reactions in the dermis was investigated after pretreatment of the sites with

Figure 1. Effect of pretreatment of dermal sites with bFGF on monocyte recruitment to inflammatory stimuli. Dorsal skin of Lewis rats was pretreated intradermally with 50 ng of bFGF and 5 μ g of heparin or heparin alone. After 2 days, ^{51}Cr -labeled monocytes were injected intravenously, and skin sites were reinjected with bFGF and heparin or heparin together with inflammatory stimuli (100 U of IFN- γ , 3 ng of TNF- α , 10% of ZAS as source of C5a, 10 μ g of *M. butyricum* for DTH reaction). After 18 hours the skin was removed, and ^{51}Cr in the dermal sites was quantitated. Values are mean percentages $\pm$ SEM of control (heparin-treated) sites, which are set as the 100% response. The mean absolute ^{51}Cr -monocyte accumulation per lesion is given under each bar pair ($n = 4$ to 11). * $P < 0.05$, *** $P < 0.005$, paired t -test.

bFGF for 48 hours. Sites on dorsal skin were pretreated by intradermal injection with 50 ng of bFGF and 5 μ g of heparin or HSA and heparin as control 48 hours before and again at the time of the injection of inflammatory stimuli. The bFGF dose selected was based on dose-response experiments between 2 and 200 ng of bFGF as indicated in Materials and Methods. The maximum response was reached with 50 ng ($163 \pm 12\%$ increase in presence of TNF- α and IFN- γ), with no further increase at doses of 100 and 200 ng of bFGF. However, even doses of 2 to 10 ng of bFGF induced increased leukocyte recruitment in response to TNF- α and IFN- γ (data not shown). Heparin was used as a co-factor at the lowest concentration (5 μ g), which avoided any effect on local hemostasis. The doses of inflammatory stimuli were selected to induce submaximal leukocyte accumulation ($\sim 50\%$ maximal for each stimulus). Figure 1 shows the recruitment of monocytes to various stimuli, normalized to 100% of the response to stimulus and HSA and heparin as the control. Pretreatment or repeated treatment of dermal sites with bFGF did not

Figure 2. Effect of pretreatment of dermal sites with bFGF on T-cell recruitment to IFN- γ . Skin sites were pretreated for 2 days as in Figure 1, and then ^{111}In -labeled T lymphocytes were injected intravenously. At the same time, the sites were reinjected with bFGF and heparin or heparin alone plus inflammatory stimuli (100 U of IFN- γ , 3 ng of TNF- α , 10 μ g of *M. butyricum* for DTH reaction). After 18 hours ^{111}In in the sites was quantitated. Values are mean percentage $\pm$ SEM of control sites expressed as in Figure 1 ($n = 4$ to 8). *** $P < 0.005$, paired t -test.

Figure 3. Effect of pretreatment of dermal sites with bFGF on PMN recruitment to inflammatory stimuli. Skin sites were pretreated as in Figure 1. After 2 days, ^{111}In -labeled PMNs were injected intravenously, and the sites were reinjected with bFGF and heparin or heparin plus inflammatory stimuli (100 U of IFN- γ , 3 ng of TNF- α , 10% ZAS for C5a, 20 ng of MIP-2). After 2 hours ^{111}In in the sites was quantitated. Values are mean percentages $\pm$ SEM of control sites expressed as in Figure 1 ($n = 4$). * $P < 0.05$, paired t -test.

induce recruitment of monocytes (Figure 1), T cells (Figure 2), or PMNs (Figure 3) in the absence of inflammatory stimuli (ie, diluent alone). However, the recruitment of monocytes in response to IFN- γ , TNF- α , TNF- α and IFN- γ , C5a, and a DTH reaction was enhanced significantly by bFGF pretreatment, ranging from 155 to 212% of control (Figure 1). The T-cell recruitment was also significantly increased in response to IFN- γ in the bFGF-pretreated sites relative to the control dermal sites (216% of control, $P < 0.001$). However, the T-cell migration in response to TNF- α and IFN- γ or DTH reactions was not modified by bFGF pretreatment (Figure 2).

We have shown that PMN recruitment is maximal within the initial 2 hours of induction of dermal inflammation³¹; therefore, a 2-hour PMN migration period was studied. A significant increase in the recruitment of PMNs to TNF- α , TNF- α and IFN- γ , and MIP-2 was observed in the sites pretreated with bFGF (232%, 195%, and 130% of control, respectively). In contrast, the PMN recruitment induced by C5a was not affected by bFGF pretreatment (Figure 3).

Enhancement of Leukocyte Recruitment to Inflammatory Stimuli Induced by bFGF Is Rapid and Persistent

Because of the unexpected results above, we investigated whether the modulating effect of bFGF treatment was time-dependent. Therefore, 50 ng of bFGF and 5 μ g of heparin was injected (intradermally) in dorsal skin sites in the following schedules: 1) daily for 5 days (days -5 to 0); 2) once, 48 hours before (day -2) the inflammatory stimulus; 3) twice, 48 hours before and at the time of inflammatory stimulus (days -2 and 0); or 4) only at the time of injection of inflammatory stimulus (day 0). Radio-labeled PMNs and monocytes were injected (day 0) and allowed to migrate for 2 hours. Figure 4, a and b, shows that daily pretreatment up to 5 days induced increased recruitment of monocytes ($203 \pm 27\%$ of control) and

Figure 4. Effect of acute versus chronic bFGF treatment of dermal sites on recruitment of leukocytes to inflammatory stimuli. Treatment of dermal sites with bFGF daily for 5 days (day -5 to 0); once, 48 hours before (day -2); twice, 48 hours before and at the time of inflammatory stimulus (days -2 and 0); or only at the time of injection of inflammatory stimulus (day 0) was compared. Inflammation with IFN- γ (**a** and **c**) or TNF- α and IFN- γ (**b**) was induced 2 hours (**a** and **b**, for monocytes and neutrophils, respectively) or 18 hours (**c**, for T cells) before sacrifice. At the same time, ^{51}Cr -monocytes and ^{111}In -PMNs or ^{111}In -T cells were injected intravenously. The ^{51}Cr and ^{111}In in the dermal sites were quantitated. Values are mean percentages $\pm$ SEM of control sites ($n = 4$ to 10). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, paired t -test.

PMNs ($171 \pm 16\%$ of control) in response to IFN- γ and TNF- α , respectively. Co-injection of bFGF together with inflammatory mediator without pretreatment (day 0) induced a similar result. These levels of increase over the control were comparable to pretreatment at days -2 and 0 used in Figures 1 and 3. However, pretreatment of sites once only 2 days before (day -2) the induction of inflammation did not affect leukocyte recruitment induced by IFN- γ or TNF- α and IFN- γ . Similarly to PMN and monocyte recruitment, T-cell recruitment in response to IFN- γ was increased to $269 \pm 46\%$ of control when bFGF was injected with IFN- γ but was not significantly different from control pretreatment when bFGF was administered at day -2 only (Figure 4c).

Rat and Human bFGF Have Comparable Effect on Leukocyte Recruitment

Rat and human bFGF share 88% sequence identity. We investigated whether rat bFGF had the same effect as human bFGF on PMN recruitment in paired sets of experiments ($n = 3$). Rat bFGF enhanced the recruitment of PMNs in response to TNF- α and IFN- γ by $132 \pm 51\%$, comparable to the increase induced by human bFGF ($166 \pm 41\%$). This indicates that our observation with human bFGF is not due to species

differences but rather to a biological effect on the inflammatory response.

The Synergistic Effect of bFGF on the Recruitment of Leukocytes Is Not Accompanied by Increased Vascular Permeability and Blood Flow

Increased vascular permeability and, as a consequence, extravasation of serum proteins are features of inflammation and can also enhance the rate of PMN emigration.⁴¹ To investigate the effect of bFGF on vascular permeability, ^{125}I -HSA extravasation from plasma was measured in the bFGF-pretreated and control dermal sites. As shown in Table 1, no difference in the extravasation of plasma albumin was observed with any of the inflammatory stimuli by bFGF or control (heparin) pretreatment.

Inflammatory hyperemia is known to enhance PMN recruitment induced by PMN recruiting mediators.⁴⁵ Therefore, the blood flow was quantified with $^{86}\text{RbCl}$ uptake. As shown in Table 1, blood flow (expressed as fold increase) was increased in response to all inflammatory stimuli tested. However, there were no differences be-

Table 1. Parameters of Vascular Permeability, Hyperemia, and Angiogenesis in bFGF-Pretreated and Control Dermal Sites

	IFN- γ	TNF- α	TNF- α + IFN- γ	C5a	DTH	Diluent
Vascular permeability*						
18 hours inflammation						
bFGF + heparin	3.6 $\pm$ 0.5	3.4 $\pm$ 0.7	3.1 $\pm$ 0.6	2.7 $\pm$ 0.1	8 $\pm$ 1 [§]	4.9 $\pm$ 0.8
Heparin	3.7 $\pm$ 0.4	2.9 $\pm$ 0.3	2.6 $\pm$ 0.5	2.3 $\pm$ 0.1	7 $\pm$ 0.7 [§]	4.6 $\pm$ 0.7
2 hours inflammation						
bFGF + heparin	9.3 $\pm$ 0.3	11 $\pm$ 1	14 $\pm$ 1.4	77 $\pm$ 5 [§]	ND	11 $\pm$ 1
Heparin	8.3 $\pm$ 0.9	8 $\pm$ 2	12 $\pm$ 0.8	67 $\pm$ 7 [§]	ND	9 $\pm$ 0.5
Blood flow [†]						
bFGF + heparin	1.9 $\pm$ 0.3	ND	1.6 $\pm$ 0.2	ND	1.9 $\pm$ 0.3	1.1 $\pm$ 0.1
Heparin	1.7 $\pm$ 0.3	ND	1.4 $\pm$ 0.2	ND	1.8 $\pm$ 0.3	1 $\pm$ 0.2
Vascular pool [‡]						
bFGF + heparin	ND	3.4 $\pm$ 0.4	3.1 $\pm$ 0.6	3.2 $\pm$ 0.4	1.5 $\pm$ 0.1	2.5 $\pm$ 0.5
Heparin	ND	3.2 $\pm$ 0.4	2.7 $\pm$ 0.4	2.9 $\pm$ 0.9	1.2 $\pm$ 0.3	2.2 $\pm$ 0.4

*Expressed as μ l plasma albumin/lesion. Treatment with bFGF was at days -2 and 0 for all lesions shown.[†]Expressed as fold of increase over the diluent control. Values measured in 2-hour-old lesions.[‡]Expressed as μ l plasma IgG/lesion.[§] $P < 0.05$ relative to diluent injected sites. Values are mean $\pm$ SEM ($n = 2$ to 10).

tween bFGF-pretreated and control sites in normal or in inflamed tissue.

The Increased Recruitment of Leukocytes to Inflamed Dermal Sites by bFGF Is Not Associated with Angiogenesis

bFGF is a potent angiogenic factor, eg, in the corneal implantation or in the Matrigel angiogenesis assays.^{46,47} We therefore examined whether the increased leukocyte recruitment induced by bFGF, especially after 2 to 5 days of bFGF pretreatment, might be associated with angiogenesis. This was assessed by labeling the intravascular blood pool in the tissues with an injection (intravenous) of ¹²⁵I-IgG⁴⁰ and by immunohistochemistry for blood vessel density enumeration in the skin sites. As shown in Table 1, there was no significant difference in the tissue vascular pool. Furthermore, the histological quantification of small, medium, and large vessels revealed no differences between control and bFGF-pretreated sites after

either 2 or 5 consecutive days of bFGF treatment (not shown). Figure 5 shows vessel staining in representative bFGF-pretreated (Figure 5a) and control (Figure 5b) sites.

The Enhanced Recruitment of PMNs to Inflammation Induced by bFGF Is Not Tissue-Dependent

We investigated whether the enhancement of PMN recruitment by bFGF was an effect observed only in dermal tissue or also in other tissues, such as joints. Inflammation was induced in ankle (talar) and wrist (carpal) joints by injection of 100 ng of TNF- α and 300 U of IFN- γ $\pm$ 50 ng of bFGF. The recruitment of ¹¹¹In-labeled PMNs was evaluated after 2 hours. As shown in Figure 6, bFGF induced significantly increased PMN recruitment to these joints in response to inflammatory stimuli (138 to 165% of control, $P < 0.05$). A similar effect was observed with rat bFGF (135 to 140% of control, $n = 4$, $P < 0.05$).

Figure 5. Histological assessment of bFGF treatment on dermal vessels. Dermal sites were treated daily for 5 days with bFGF (50 ng of bFGF and 5 μ g of heparin) (a) or control (heparin alone) (b) and then paraffin-embedded, and 5- μ m sections were stained for laminin to identify blood vessels. Antibody binding was detected indirectly with biotin-SP-conjugated goat anti-rabbit IgG and developed with a ABC-DAB kit. Original magnifications, $\times 200$.

Figure 6. Effect of bFGF on PMN recruitment to joints induced by TNF- α and IFN- γ . Intra-articular injections (20 μ l) of 50 ng of bFGF, 100 ng of TNF- α , and 300 U of IFN- γ in diluent (RPMI-HSA-heparin) were administered, followed by intravenous injection of 111 In-labeled PMNs. After 2 hours, the 111 In in the joints was quantitated. Values are mean percentages $\pm$ SEM of the response in the contralateral control joints injected with the above combination without bFGF ($n = 24$ to 32). *** $P < 0.005$, paired t -test.

Expression of CAMs in TNF- α - and IFN- γ -Inflamed Skin Is Enhanced by bFGF

To elucidate the mechanisms by which bFGF induces an increase in the recruitment of leukocytes in response to inflammatory mediators, we quantified the expression of adhesion molecules in the dermal sites as shown in Figure 7. It was noted that diluent injection alone induced a slight but significant increase in P-selectin expression ($P < 0.01$) but not E-selectin, ICAM-1, or VCAM-1 when compared to noninjected skin (NS; not shown but values subtracted). Various lipopolysaccharide-free diluents were tested (Tyrode's buffer, RPMI) with and without HSA without any effect on the small degree of P-selectin increase compared to

Figure 7. Effect of bFGF on adhesion molecule expression in inflamed dermal sites. The dorsal skin of Lewis rats was injected intradermally with 3 ng of TNF- α and 100 U of IFN- γ $\pm$ 50 ng of bFGF and 5 μ g of heparin, with diluent (RPMI-HSA-heparin) or with bFGF and heparin. After 2 hours, 25 μ g of 125 I-labeled anti-ICAM-1 mAb (1A29), anti-VCAM-1 mAb (5F10), anti-P-selectin mAb (RMP-1), or anti-E-selectin mAb (RME-1) and 25 μ g of 131 I-labeled isotype control IgG were injected intravenously and allowed to circulate 5 minutes. The animals were then sacrificed, the skin sites removed, and 125 I and 131 I quantitated. CAM expression is expressed as the mean $\pm$ SEM of the specific anti-CAM mAb bound in the site [percentage of injected dose (ID) of anti-CAM mAb - percentage of ID of isotype control mAb (131 I)] - value of 125 I in noninjected skin; NS, $n = 13$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired t -test.

noninjected skin. This P-selectin increase may reflect some degree of EC activation by, for example, mast cell degranulation secondary to the trauma of intradermal injection (even though only a 30-gauge needle was used) and/or platelet P-selectin associated with platelet deposition in a hemostatic plug. Injection of bFGF induced a significant increase in the expression of ICAM-1 and P-selectin in the absence of inflammatory cytokines (131% and 67% increase, respectively). TNF- α and IFN- γ also significantly increased the expression of VCAM-1, P-selectin, and E-selectin relative to the diluent control. Co-injection of bFGF (and heparin) together with TNF- α and IFN- γ enhanced ICAM-1 expression ($P < 0.05$) when compared to TNF- α and IFN- γ (and heparin as control). Furthermore, bFGF also significantly increased P- and E-selectin expression in TNF- α and IFN- γ -stimulated sites. The expression of VCAM-1 showed an increasing tendency in bFGF- and TNF- α - and IFN- γ -stimulated sites, although due to variability, this did not reach significance (Figure 7).

It was considered that the enhanced expression of CAMs observed in bFGF-co-treated sites during inflammation may be a secondary effect due to the increased leukocyte recruitment. To assess this potential mechanism, we blocked the emigration of all leukocyte types by intravenous injection of anti-CD18 and anti-CD49d antibodies as shown previously^{38,42,43} and measured P- and E-selectin expression in skin sites after TNF- α and IFN- γ and bFGF injection. These experiments showed that the higher P- and E-selectin expression in bFGF-co-treated sites was not altered despite essentially complete blockade of PMN recruitment to the tissue, which was simultaneously measured (for the control rat group, TNF- α and IFN- γ and bFGF sites, the specific CAM expression in net percentage $\times 10^{-3}$ for E-selectin was 3.4 ± 0.5 , and 5.2 ± 0.3 for P-selectin, and PMN accumulation was 2417 ± 269 cpm/site; for the anti-CD18 and anti-CD49d mAb-treated group in TNF- α and IFN- γ and bFGF sites, expression of E-selectin was 2.7 ± 0.5 and of P-selectin was 4.6 ± 0.2 , and PMN accumulation was 35 ± 4 cpm; $n = 3$). This indicates that bFGF-enhanced CAM expression in response to the inflammatory cytokines used is independent of the leukocyte infiltration.

Figure 8 shows representatives of 190 vessels examined by immunofluorescence staining for E-selectin, P-selectin, and ICAM-1 expression. Blinded observer assessment indicated an increase in the percentage of positive-staining postcapillary venules as well as an increase in the intensity of staining with anti-E- and anti-P-selectin antibodies in the sites treated with bFGF and TNF- α and IFN- γ when compared with TNF- α and IFN- γ alone, as seen also in the figure. ICAM-1 staining was strong under all conditions, and differences between bFGF-treated and nontreated inflamed sites were difficult to appreciate visually, although quantitation by radiolabeled anti-ICAM-1 mAb binding indicated a small but significant increase in ICAM-1 expression when bFGF was administered with TNF- α and IFN- γ as indicated above.

Figure 8. Immunofluorescence detection of E-selectin, P-selectin, and ICAM-1 on dermal vessels. Inflamed dermal sites (TNF- α and IFN- γ $\pm$ bFGF) were paraffin-embedded, and 5- μ m sections were stained for E-selectin (14G2 mAb), P-selectin (RP-2 mAb), or with an isotype control mAb. For ICAM-1 detection, 8- μ m frozen sections were stained with 1A29 mAb or isotype control. A secondary goat anti-mouse IgG biotin-conjugated antibody and further development with streptavidin-Alexa Fluor 568 detected the primary antibody binding. Representative bright field and fluorescence fields in gray scale are shown. Original magnifications, $\times 200$.

Discussion

Infection or inflammatory cytokines have been reported to induce bFGF synthesis.^{48,49} bFGF is produced by several cell types, including ECs and fibroblasts.^{50–52} Released bFGF may be stored in the extracellular matrix by binding to heparan sulfate proteoglycans, and heparinases have a role in the release of bFGF from connective tissue stores.^{8,53} bFGF is one of the most potent stimulators of angiogenesis and is mitogenic and chemotactic for both ECs and fibroblasts. On ECs bFGF has been reported to down-regulate the expression of CAM and the interaction of leukocytes with ECs *in vitro*. Extending these observations *in vivo*, we systematically examined the effects of bFGF on the vascular responses and leukocyte recruitment during inflammation.

The first notable finding in this study is that bFGF (with or without heparin) did not induce leukocyte recruitment or any vascular response of inflammation (permeability or hyperemia) after either acute or repeated administration into the dermis. This finding is consistent with that of

Yamashita and colleagues²⁹ that transfecting bFGF in a recombinant Sendai virus vector delivered in rat ankle joints did not induce inflammation. Similarly, bFGF transgenic mice with increased endogenous levels of bFGF did not have spontaneous inflammation.²⁸ Previous *in vitro* results in our laboratory demonstrated that bFGF treatment of HUVECs for 24 hours or longer downmodulated the transendothelial migration of monocytes and PMNs to a variety of stimuli including cytokines, suggesting that bFGF may have an anti-inflammatory role.^{22,23} This prompted us to investigate the effect of bFGF on various types of inflammation *in vivo*. Our findings indicated that bFGF, either acutely or on repeated application, enhanced the recruitment of monocytes in response to a variety of inflammatory mediators including cytokines, chemotactic factors, or to the DTH reaction (Figure 1). The synergizing effect of bFGF was observed also in the recruitment of T cells to IFN- γ and acute PMN recruitment within the first 2 hours to TNF- α , TNF- α and IFN- γ , and to a lesser extent, MIP-2-induced inflammation (Fig-

ures 2 and 3). The effect of bFGF observed here in dermal inflammation is not tissue-specific, because bFGF also acutely increased the recruitment of PMNs (within 2 hours) into normal joints in response to TNF- α and IFN- γ (Figure 6). Furthermore, rat bFGF had a similar enhancing effect to that of human bFGF on leukocyte recruitment to both skin and joint inflammation indicating that the observed effects are not unique to human bFGF in the rat. These results indicate that bFGF has a rapid and synergistic effect in heightening leukocyte responses to some inflammatory cytokines and that *in vivo*, even repeated or prolonged bFGF exposure of vascular endothelium and tissues does not alter this leukocyte recruitment-enhancing action. Our direct and controlled studies with respect to various stimuli, duration, and quantification of recruitment of various leukocyte types extends the *in vivo* findings of Yamashita and colleagues²⁹ in rat adjuvant arthritis indicating that bFGF overexpression in arthritic joints aggravates inflammation. Similarly, Meij and colleagues²⁸ observed exacerbation of inflammatory myocardial injury and T-cell infiltration in bFGF-overexpressing mice.

Inflammatory hyperemia and increase in vascular permeability are microvascular responses during the acute inflammatory reaction. These parameters can have a modulating effect on the rate of PMN recruitment.^{41,45} As shown in Table 1, bFGF (and heparin) alone had no effect on these vascular responses. We used submaximal concentrations of inflammatory stimuli to allow detection of modulation of the responses by bFGF in either direction. As shown in Table 1, these submaximal concentrations of inflammatory factors were sufficient to induce an increase in the blood flow, although the vascular permeability did not measurably change compared to nonstimulated sites with the exception of the most potent stimulus for PMN recruitment, namely C5_{ades-arg} (ZAS). Our results demonstrate that the effect of bFGF on inflammatory stimulus-induced leukocyte recruitment is not due to modulation of the blood flow or vascular permeability responses.

As mentioned above, bFGF is a potent angiogenic factor that is increased in inflamed tissues, eg, in inflammatory bowel disease²⁴ and in synovial fluid in severe rheumatoid arthritis.⁵⁴ In these pathologies there is chronic inflammation and active angiogenesis. Although angiogenesis could affect the surface area for leukocyte emigration into inflamed tissue, our results show that bFGF did not induce angiogenesis in normal, uninfamed skin, even after daily treatment of dermal tissue for 5 days (Table 1 and text; Figure 5). Thus, the observed enhancement of leukocyte recruitment by prolonged, repeated treatment was not secondary to angiogenesis. Furthermore, the acute effect of bFGF in enhancing leukocyte recruitment within 2 hours of administration, when combined with the inflammatory stimulus, suggests that the action of bFGF is acute and independent of angiogenesis. Growth of new blood vessels in adult skin in the absence of injury or inflammation is not detectable. bFGF-induced angiogenesis *in vivo* has been reported in the chick chorioallantoic membrane assay, corneal implantation assay, and the Matrigel plug assay.⁵⁵ The chick chorioallantoic membrane assay is an embryolog-

ical and developmental system in which other angiogenic factors are also involved. In the Matrigel plug assay, an inflammatory reaction surrounding the plug does occur. In this assay we have observed that the bFGF used here induced angiogenesis in the plug in rats (unpublished observations), even though direct bFGF injection intradermally did not. In the corneal implant assay, some foreign body irritant and local trauma may generate inflammatory stimuli, which could potentiate the angiogenic action of bFGF. Thus, although bFGF is clearly angiogenic in pathological settings where other factors may contribute to this action, our findings indicate that bFGF has independent effects in enhancing inflammatory and immune cell recruitment, which chronically could lead to an angiogenic environment.

Our findings indicate that one mechanism for the acutely enhanced leukocyte recruitment induced by bFGF to inflammation is the up-regulation of CAM expression. Within 2 hours of TNF- α and IFN- γ injection, the expression of ICAM-1, VCAM-1, E-selectin, and P-selectin increased over noninjected skin, and this was significantly enhanced by co-administration of bFGF for ICAM-1, P-selectin, and E-selectin (Figure 7). This increased expression of E- and P-selectin induced by bFGF was confirmed by immunofluorescence (Figure 8). This was not a secondary effect of the accumulated leukocytes stimulating enhanced CAM expression, because when the recruitment of PMNs was blocked by >98%, P- and E-selectin expression was still increased by bFGF treatment (see Results). We have observed similar degrees of inhibition of monocyte and lymphocyte recruitment to skin inflammation using anti-CD18 and anti-CD49d mAbs.^{38,42,43} Thus, the mechanism of bFGF potentiation of CAM expression in response to TNF- α and IFN- γ is likely to be local at the level of the endothelium or perhaps secondary via an effect on other resident cells, which may co-stimulate the microvascular endothelium. Because P-selectin and E-selectin are critical adhesion molecules for the capture and rolling of leukocytes in the microvasculature and ICAM-1 mediates firm adhesion, the up-regulation of these CAMs associated with bFGF during inflammation may well result in more efficient leukocyte emigration into the surrounding tissues. Whether this is the only mechanism modulated by bFGF remains to be determined.

The literature reports differing effects of bFGF on EC-leukocyte interactions. *In vitro* and *in vivo* studies have shown a reduced expression of endothelial adhesion molecules such as ICAM-1, ICAM-2, and CD34.^{18,20,56,57} Results from our laboratory using HUVECs have shown that bFGF treatment for 18 hours transiently increased expression of ICAM-1 and VCAM-1, but not E-selectin, whereas treatment for more than 24 hours downmodulated the expression of ICAM-1, VCAM-1, and MCP-1 protein production.^{22,23} Griffioen and colleagues²⁰ also demonstrated inhibition by 40 to 70% of CAM expression on HUVECs pretreated with bFGF for 3 days. These results are in concordance with observations in tumors,^{15,17} where the rolling and adherence of leukocytes to the angiogenic vasculature were found to be diminished. However, what role bFGF or other factors and the

neovessels played in this observation was not determined. Our results *in vivo* with bFGF in normal, nonangiogenic tissue cannot be directly compared with these *in vitro* (HUVECs) and tumor-vessel studies because the ECs under these conditions are not quiescent but in growth phase. Tromp and colleagues⁵⁸ observed inhibition of leukocyte adhesion to ECs in cremaster muscle vessels activated with IL-1 β for 3 hours after 3 days of exposure to slow-release pellets of bFGF. The state of the vessels analyzed, ie, normal or angiogenic, was not mentioned, but the bFGF pellets did induce angiogenesis in skinfold chambers. Furthermore, the surgical placement of alginate pellets in the scrotum may cause inflammation, which, after 3 days and under the exposure to bFGF, might affect the vascular responsiveness to IL-1 β . Thus, the tissue, stimulus and possibly the vascular state were different from that in our studies. Some of these differences could explain the contrasting results.

In summary, we demonstrate that bFGF has direct and acute effects on normal tissue to synergistically enhance recruitment of PMNs, monocytes, and T cells in response to inflammatory cytokines and DTH reactions independent of angiogenesis. Many conditions characterized by leukocyte infiltration are associated with both angiogenesis and the presence of bFGF in the tissue.^{24,54,59} Our results are the first to show that bFGF has acute effects in inflammation *in vivo* and that this is associated at least in part by potentiation of CAM up-regulation on ECs *in vivo* in response to inflammatory cytokines. Taken together with recent findings in arthritis and myocardial inflammation, bFGF may be an important positive regulator of leukocyte recruitment in acute and chronic inflammation.

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