Brief Definitive Report

Development of Inflammatory Angiogenesis by Local Stimulation of Fas In Vivo

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Summary

Fas–Fas ligand interaction is thought to be a crucial mechanism in controlling lymphocyte expansion by inducing lymphocyte apoptosis. However, Fas is also broadly expressed on nonlymphoid cells, where its function in vivo remains to be determined. In this study, we describe the development of inflammatory angiogenesis induced by agonistic anti-Fas mAb Jo2 in a murine model where Matrigel is used as a vehicle for the delivery of mediators. The subcutaneous implants in mice of Matrigel containing mAb Jo2 became rapidly infiltrated by endothelial cells and by scattered monocytes and macrophages. After formation and canalization of new vessels, marked intravascular accumulation and extravasation of neutrophils were observed. Several mast cells were also detected in the inflammatory infiltrate. The phenomenon was dose and time dependent and required the presence of heparin. The dependency on activation of Fas is suggested by the observation that the inflammatory angiogenesis was restricted to the agonistic anti-Fas mAb and it was absent in Ipr Fas-mutant mice. Apoptotic cells were not detectable at any time inside the implant or in the surrounding tissue, suggesting that angiogenesis and cell infiltration did not result from recruitment of phagocytes by apoptotic cells but rather by a stimulatory signal through Fas engagement. These findings suggest a role for Fas-Fas ligand interaction in promoting local angiogenesis and inflammation.

Fas and structurally related glycoproteins form a family of surface receptors that control cell proliferation, survival, or apoptosis (1). On the other side, their ligands are homologue trimers present on soluble and/or cell-associated forms that may act as growth or death factors for several cell types. In particular, Fas ligand (FasL) is expressed on the cell surface and released by T cells (2, 3), monocytes, and neutrophils (4). So far, the biological importance of Fas-FasL interaction has been investigated in T lymphocytes, where it controls clonal deletion of autoreactive lymphocytes and mediates activation-induced suicide of T cells through a death signal transduced by Fas (1). However, several cell types, such as endothelial cells (5), monocytes (4), epithelial cells (6), and certain tumor lineages (7), are resistant to Fas-mediated apoptosis. Other cells, such as neutrophils (4) and B cells (8), rapidly acquire resistance after stimulation with growth factors or cytokines. In addition, genetic deficiency of Fas or FasL does not affect organ development with the exception of the lymphoid compartment. Therefore, the role of Fas-FasL interaction in nonlymphoid cells remains to be elucidated, and it might also involve mechanisms different from apoptosis induction. Indeed, this hypothesis is fueled by an increasing number of observations suggesting that Fas may transduce cell activation signals independently or as an alternative to cell death (9–12).

This study explores the effect of an in situ Fas stimulation by agonistic monoclonal antibodies implanted in vivo within Matrigel. In this model, anti-Fas mAb triggers neoangiogenesis and local infiltration of inflammatory cells independently from apoptosis.

Materials and Methods

Reagents. Hamster anti–mouse Fas Jo2 was from PharMingen (San Diego, CA) and rat anti–mouse Fas RMF6 was from MBL (Nagoya, Japan). Purified hamster IgGs (Cappel Laboratories Inc., West Chester, PA) were used as control.

Murine Angiogenesis Assay. Wild-type and carrier of the Ipr mutation in the Fas gene C57Bl/6 (13) mice were from Jackson Laboratories (Bar Harbor, Maine). Purified hamster IgGs (Cappel Laboratories Inc., West Chester, PA) were used as control.

M urine Angiogenesis Assay. Wild-type and carrier of the Ipr mutation in the Fas gene C57Bl/6 (13) mice were from Jackson Laboratories (Bar Harbor, Maine). Animals were used at 6 wk of age. Angiogenesis was assayed as growth of blood vessels from subcutaneous tissue into a solid gel of basement membrane containing the test sample (14). 6 mice per condition in each experiment were used. As standard procedure, Matrigel (8.13 mg/ml; Becton Dickinson Labware, Bedford, MA), in liquid form at 4°C, was mixed with heparin (64 U/ml, Sigma Chem. Co., St. Louis, MO) and the experimental substances and injected (0.25 ml) into the abdominal subcutaneous tissue of mice, along the peritoneal midline. At various times, mice were killed and gels were processed for light microscopy, detection of nonspecific esterase activity, and immunohistochemistry as previously described (15). Rabbit anti-vWF (Sigma), anti-VEGF, anti-flk-1 and anti-b-FGF

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(Santa Cruz Biotechnology, Santa Cruz, CA), and anti-L3/T4 and Ly2 monoclonal antibodies (Cedarlane, Ontario, Canada), as well as control rabbit and mice IgG, were used as primary antibodies for indirect immunofluorescence. FITC-conjugated anti-rabbit IgG and anti-rat IgG were used as secondary antibodies (Cappel Laboratories). Direct immunofluorescence staining was performed with anti-MAC-1 FITC-conjugated monoclonal antibodies (Cedarlane). Mast cells were stained metachromatically with 1% Toluidine blue, pH 3.5, for 30 min. Apoptotic cells were detected in situ through the staining of DNA fragments with the TUNEL assay (ApoTag Oncor, Gaithersburg, MD) (16). Tissue from rat regressing mammary glands obtained at the fourth day after weaning was used as positive control for the technique.

Vessel area and the total Matrigel area were planimetrically as-

Figure 1. Histological and immunohistochemical analysis of Matrigel implants. Representative, hematoxylin-eosin-stained section of Matrigel containing 5 μg/ml anti-Fas mAb Jo2 (A) implanted in wild-type mice and excised 6 d after injection (6 mice per condition in each experiment). Progressive invasion of cells into the Matrigel implant and formation of capillaries is observed (×200). (B) Control: mAb Jo2 was replaced by hamster IgG (×200). Similar results were obtained by using nonagonistic anti-Fas mAb RMF6 or by injecting Matrigel with mAb Jo2 into lpr Fas-mutant mice (not shown). Kinetic of anti-Fas mAb Jo2-induced inflammatory angiogenesis (×400); (C) Initial migration of endothelial cells aligning to form cord-like structures at day 1; (D) appearance of PMN inside the lumen of neo-capillaries (day 2); (E) marked extravasation of PMN throughout the neoformed capillary into the Matrigel (day 6); (F) metachromatic staining by Toluidine blue of mast cells infiltrating Matrigel at day 6 (×600); (G) formation of canalized vessels containing erythrocytes and leukocytes (day 4–6). Immunohistochemical detection of apoptotic cells by the TUNEL method (normal nuclei are counterstained with propidium iodide in red); (H) Section of Matrigel with anti-Fas mAb Jo2 at day 6, displaying total absence of apoptotic cells among the infiltrating cells (×200). The same results were obtained on Matrigel excised 6 h, 12 h, 24 h, 5 d, and 10 d after injection (not shown); (I) Rat regressing mammary glands obtained at the fourth day after weaning showing apoptotic cells (arrows) along the acini, as positive control (×200).
Results

Subcutaneous injection in mice of Matrigel containing the agonistic anti-Fas mAb Jo2 caused rapid neovascularization and infiltration of inflammatory cells within the implant (Fig. 1). The phenomenon is dose and time-dependent (Figs. 2 and 3). The minimal effective dose was 100 ng/ml. Initial infiltration of cells was observed as early as 24 h after injection (Fig. 2 A) and the infiltrating cells were mainly endothelial cells (Fig. 1 A), as detected by immunofluorescence staining for vWF (Fig. 4, A and B) and MAC-1+ (Fig. 4 C) and aspiric esterase plus monocytes. Polymorphonuclear neutrophils (PMN) were seen initially at the periphery of Matrigel implant, then within the lumen of neoformed capillary sprouts (Fig. 1 D) and finally around the neoformed vessels (Fig. 1 F). Only scattered lymphocytes CD4+ or CD8+ were detected. Several mast cells (Fig. 1 F), metachromatically stained by Toluidine blue, were also detected within the Matrigel and the inflammatory infiltrate surrounding the neoformed vessels. Maximal angiogenesis was observed at day 6 with formation of canalized vessels (Fig. 1 G and 3 A). At that time, ~50% of neoformed vessels were surrounded by an intense infiltration of inflammatory cells similar to granulation-like tissue (Fig. 1 E). Control purified hamster IgG and a non-signaling anti-mouse Fas mAb RMF6 (17) were completely ineffective (Fig. 5). mAb Jo2 was endotoxin free as tested by Limulus assay and in selected experiments prein-
Incubation of this tissue without TdT enzyme, as negative control, resulted in absence of staining.

**Discussion**

Interaction of Fas with its ligand, FasL, or agonistic antibodies may lead to induction of intracellular pathway of apoptosis. However, while this phenomenon is easily observable on activated lymphocytes (1) and certain tumor lineages (7), it does not affect several Fas-expressing cell types such as endothelial (5), monocytes (4), eosinophils (4), and epithelial cells (6), or it is prevented by a number of environmental survival signals (e.g., growth factors, extracellular matrix, cell-to-cell interaction molecules, antigens) in cells such as neutrophils (4) and B-lymphocytes (8). In addition, Fas and FasL mutant MRL mice that develop a spontaneous autoimmune disorder due to failure in deleting lymphocytes, do not show tissue-specific alterations unrelated to the autoimmune process (18). These observations seem to limit the physiologic apoptotic effect of Fas to the immune cell compartment.

Recently, an increasing amount of evidence suggest that Fas may transduce also cell-activation signals independently or in alternative to cell death (9-12). Therefore, in vivo Fas may potentially have alternative functions as observed for other members of its family including TNF-receptors (19), and CD40 (20). All these molecules may indeed trigger activation and proliferation or, conversely, apoptosis in distinct cell types or in different experimental conditions.

This study provides evidence of a role of Fas in promoting angiogenesis and inflammatory cell recruitment in vivo.
In mice subcutaneous implants of Matrigel containing anti-Fas mAb are rapidly neovascularized and infiltrated by inflammatory cells. Neoangiogenesis is triggered also by VEGF (21), b-FGF (14), TNF-α (15), and PAF (15), however, no inflammatory infiltration is caused by these agents. Therefore, the inflammatory angiogenesis observed in the present study is peculiar of Fas-stimulation. Indeed, a nonsignaling anti-Fas mAb (17) failed in inducing this effect, suggesting that anti-Fas mAb does not act simply by anchoring or facilitating the interaction of Fas-expressing cells with the Matrigel. In addition, the role of a functional activation of Fas is suggested by the absence of inflammatory angiogenesis in Fas-deficient mice stimulated with agonistic anti-Fas mAb. 

A time course analysis of apoptosis did not reveal the presence of apoptotic cells in the surrounding tissue or inside the Matrigel containing the anti-Fas mAb, suggesting that inflammatory cell accumulation cannot be ascribed to the recruitment of phagocytes by apoptotic cells. Cell necrosis was not observed.

Interestingly, in a model of xenotransplantation, FasL expression by a graft implanted subcutaneously in nude mice elicited a rejection reaction which was mediated by neutrophil recruitment and activation (22). Our results demonstrate that in the subcutaneous site Fas-stimulation induces per se a marked angiogenic and inflammatory response. The local prevalence of cells naturally resistant to Fas-mediated apoptosis and/or the availability of survival signals, such as growth factor supply and extracellular matrix interaction, may account for the lack of apoptosis in this model. Therefore, it is conceivable that Fas stimulation by agonistic mAb may uncover in this model the cell activation rather than the apoptotic signals. Furthermore, the observation that heparin was required for Fas-induced inflammatory angiogenesis, suggests that heparin-binding growth factors may be involved as secondary mediators. The immunohistochemical study revealed the expression of VEGF and its receptor Flk-1 by the endothelial cells infiltrating Matrigel or lining the neoformed vessels. In the present experimental model, multiple cell types may act as a target for anti-Fas antibodies. However, the early infiltrating cells were endothelial cells and monocytes. The time-course studies showed that the recruitment of PMN follows that of endothelial cells. When the vessels are canlized PMN were seen adherent to the abluminal surface of endothelial cells and in the extravascular space around the neoformed vessels, suggesting a leukocyte recruitment dependent on endothelial cell activation. Neutrophils constitutive express both Fas and its ligand and in vitro they become resistant to Fas-mediated apoptosis when cultured in the presence of G-CSF, GM-CSF, IFN-γ, TNF-α, or dexamethazone (4). In addition, the deficiency of Fas in Ipr mice seems to impair the inflammatory response involving polymorphonuclear leukocytes extravasation in the course of host defense against bacteria (23).

In conclusion, Fas–FasL interaction is likely to occur at the sites of inflammation because FasL may be expressed by several inflammatory cells such as T cells, monocytes and neutrophils (4). Furthermore, FasL may also be released by proteolytic cleavage as a biologically active soluble form (3, 24), which is detectable in several pathologic conditions (24), including synovial fluids of patients with rheumatoid arthritis (Biancone, L., and G. Camussi, unpublished observation). Such a proteolytic process seems to be catalyzed by metalloproteinases that are usually overproduced during inflammation (24).

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