Promotion of Mouse Fibroblast Collagen Gene Expression by Mast Cells Stimulated Via the $Fc_\epsilon RI$. Role for Mast Cell–derived Transforming Growth Factor β and Tumor Necrosis Factor α

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Summary

Chronic allergic diseases and other disorders associated with mast cell activation can also be associated with tissue fibrosis, but a direct link between mast cell mediator release and fibroblast collagen gene expression has not been established. Using in situ hybridization, we show that the elicitation of an IgE-dependent passive cutaneous anaphylaxis (PCA) reaction in mice results in a transient, but marked augmentation of steady state levels of type α-1 (I) collagen mRNA in the dermis. While peak levels of collagen mRNA expression in the skin are observed 16–24 h after mast cell activation, substantial numbers of dermal cells are strongly positive for collagen mRNA at 1 and 2 h after antigen challenge, before circulating inflammatory cells are recruited into the tissues. Furthermore, experiments in mast cell–reconstituted or genetically mast cell–deficient $WBB6F_1$-W/W^v mice demonstrate that the increased expression of collagen mRNA at sites of PCA reactions is entirely mast cell dependent. In vitro studies show that the supernatants of mouse serosal mast cells activated via the $Fc_\epsilon RI$ markedly increase type α-1 (I) collagen mRNA levels in mouse embryonic skin fibroblasts, and also upregulate collagen secretion by these cells. The ability of mast cell supernatants to induce increased steady state levels of collagen mRNA in mouse skin fibroblasts is markedly diminished by absorption with antibodies specific for either of two mast cell–derived cytokines, transforming growth factor β (TGF-β1) or tumor necrosis factor α (TNF-α), and is eliminated entirely by absorption with antibodies against both cytokines. Taken together, these findings demonstrate that IgE-dependent mouse mast cell activation can induce a transient and marked increase in steady state levels of type α-1 (I) collagen mRNA in dermal fibroblasts and that mast cell–derived TGF-β1 and TNF-α importantly contribute to this effect.

Mast cell activation induces many of the acute changes observed in IgE-dependent allergic responses (reviewed in 1, 2). However, mast cells may also contribute importantly to certain later consequences of these reactions (1, 2). In the mouse, for example, mast cells are essential for virtually all of the leukocyte infiltration that is observed during the late phase component of IgE-dependent passive cutaneous anaphylaxis (PCA)[1] reactions (3). And in this IgE-dependent reaction, mast cell activation leads to leukocyte recruitment at least in part via the production of TNF-α (3).

While several lines of evidence now suggest that mast cells can promote leukocyte recruitment in many different settings (1), the contribution of the mast cell to other important changes that are observed in chronic allergic disorders is more obscure. For example, allergic asthma is associated not only with the infiltration of the affected tissues with leukocytes, but also with the prominent development of increased amounts of collagen and other extracellular matrix proteins beneath the basement membranes of the respiratory epithelium (4). Fibrosis also can occur in the skin of patients with atopic dermatitis (5). A variety of other conditions that are associated with evidence of mast cell activation, but that apparently do not involve IgE antibodies, are also characterized by pathologic deposition of fibrous tissue. Such conditions include chronic graft-vs.-host disease, scleroderma, Crohn's disease, and several disorders associated with pulmonary fibrosis (6, 7).

Based on these and other findings, it has been suggested

[1] *Abbreviations used in this paper:* BMCMC, bone marrow–derived cultured mast cells; NRS, normal rabbit sera; PCA, passive cutaneous anaphylaxis.

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that mast cells may promote the fibroblast activation and fibrosis associated with a wide range of allergic and nonallergic conditions that are characterized by evidence of mast cell proliferation and/or activation (6, 7). Remarkably, however, proof of a direct link between mast cell activation and the induction of fibroblast collagen production has remained elusive. In vivo studies have been inconclusive because mast cell activation often occurs in the context of complex biological responses involving many other cell types that may also influence fibroblasts. And the approaches which have been used to investigate mast cell–fibroblast interactions in vitro have focused primarily on mechanisms by which mast cells might influence fibroblast proliferation. For example, certain preformed mediators of mast cells, such as histamine (8) or tryptase (9), can induce fibroblast chemotaxis or proliferation in vitro, and leukotriene C_4 (LTC_4), a lipid mediator produced by some mast cell subsets upon IgE-dependent activation, can induce proliferation of indomethacin-treated skin fibroblasts in vitro (10). Another preformed mediator of mast cells, heparin, has been reported to be both mitogenic (11) and growth inhibitory (12) for fibroblasts. IL-4 also reportedly can induce fibroblast proliferation in vitro (13). Finally, long-term coculture of mouse 3T3 fibroblasts with immature, in vitro–derived mouse mast cells resulted in fibroblast proliferation and, in parallel, increased levels of collagen (14). However, in those studies, the changes in collagen levels were thought to reflect the effect of the mast cells on fibroblast numbers, not an effect on collagen synthesis per se (14).

While these and other studies suggest several mechanisms by which mast cells might influence fibroblast proliferation, three considerations prompted us to explore whether mast cells activated via the $Fc_\epsilon RI$ might also have effects on fibroblast collagen gene expression. First, even if the effects of individual mast cell mediators on fibroblast proliferation that have been identified in in vitro studies can also occur as a result of mast cell activation in vivo, the effects of these mediators, or mast cell activation, on fibroblast collagen production might be quite different. For example, certain mediators have effects on fibroblast proliferation and fibroblast collagen synthesis that are quite distinct. Thus, heparin can decrease proliferation but increases collagen synthesis (11), platelet-derived growth factor can increase proliferation without influencing collagen synthesis (15), and melanoma growth-stimulating activity (MGSA)/GRO has no effect on proliferation but decreases collagen synthesis (16).

Second, it is now clear that mast cells can produce several cytokines (reviewed in 17), some of which have effects on fibroblast gene expression and protein synthesis. Both in vitro–derived mouse mast cells and freshly isolated mouse serosal mast cells produce TNF-α (18, 19) and in vitro–derived IL-3–dependent or –independent mouse mast cells (20) or canine mastocytoma cells maintained in vitro (21) contain TGF-β1 mRNA; canine mastocytoma cells also can secrete TGF-β1 after stimulation with phorbol esters (21). Both TNF-α and TGF-β1 are potent activators of fibroblast interstitial matrix production (reviewed in 22). On the other hand, it has not been reported whether normal mature mast cells can produce and release TGF-β1, or whether the amounts of TNF-α and TGF-β1 that can be secreted by mast cells are sufficient to influence fibroblast collagen expression.

Finally, it is now possible to compare the expression of biological responses in vivo in the tissues of normal mice, congenic genetically mast cell–deficient W/W^v mice, and mast cell–deficient W/W^v mice that have been selectively repaired of their mast cell deficiency (3, 17, 23). This system thus permits a direct analysis of the effects of mast cell activation on dermal fibroblast collagen gene expression in vivo.

Materials and Methods

Mice

BALB/c mice were purchased from the Animal Resources Centre of the University of Saskatchewan. Genetically mast cell–deficient $WBB6F_1$-W/W^v and the congenic normal (-+/+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal treatments were performed while the mice were lightly anaesthetized with methoxyfluorane, according to the "Guidelines For the Care and Use of Experimental Animals" established by the Canadian Council on Animal Care. The mice were killed with methoxyfluorane.

Cytokines and Antisera

We purchased recombinant mouse TNF-α (rTNF-α; Genzyme Corp., Cambridge, MA), purified human TGF-β1 (R&D Systems, Inc., Minneapolis, MN), and affinity isolated chicken anti–porcine TGF-β1 IgG antibodies (R&D Systems, Inc.). Normal rabbit sera (NRS) and rabbit anti-TNFα antisera were prepared as previously described (18). The IgG was purified from these by affinity chromatography on Avid AL (BioProbe International, Inc., Tustin, CA) columns according to the manufacturer's recommendations and the protein concentrations of the eluted IgG were determined using a Coomassie Blue assay (Bio-Rad Laboratories, Mississauga, Ont., Canada). The purified anti-TNF IgG was titrated against rTNF-α and was determined to have a neutralizing capacity of $\sim 10^5$ neutralizing U/ml. One unit of activity is that amount of sample required to lyse 50% of the TNF-susceptible target cells in the L929 cell cytotoxicity assay (see below).

Cells

The derivation and/or maintenance of the growth factor–independent mouse mast cell line Cl.MC/C57.1, primary cultures of growth factor–dependent mouse bone marrow–derived, cultured mast cells (BMCMC), and the L929 cells have been reported (18). Mv1Lu mink lung cells (cell line CCL64; American Type Culture Collection [ATCC], Rockville, MD) were obtained from Dr. B. Grahn (University of Saskatchewan) and were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (GIBCO BRL), 10 mM L-glutamine, 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO), and 1% antibiotic/antimycotic (GIBCO BRL), as recommended by the ATCC. Connective tissue mast cells were purified to >99% purity (as assessed by Giemsa and/or neutral red staining) from the peritoneal cavities of $WBB6F_1$-+/+ or BALB/c mice, as reported previously (18). BALB/c 3T3 fibroblasts, generously provided by J. Gilchrist (VIDO, Saskatoon, Canada), were originally obtained from the ATCC and were maintained as recommended by the ATCC.

Mouse embryonic skin fibroblasts were generated from 13-day BALB/c embryos. The adherent outgrowth cells from the minced skin were cultured in DMEM supplemented with 10% FCS, 2.5% essential amino acids mixture (GIBCO BRL), 10 mM L-glutamine, 5×10^{-5} M 2-ME, and 1% antibiotic/antimycotic. The cells were passaged using trypsin/versene, maintained in a subconfluent state and expanded for 10 passages before being cryopreserved in liquid nitrogen. After thawing, the cells were maintained in RPMI 1640 (GIBCO BRL), 10% FCS, 10 mM L-glutamine, 5×10^{-5} 2-ME, and 1% antibiotic/antimycotic (RPMI–10% FCS) and then, just before each experiment, they were transferred to RPMI 1640, 1% FCS (or 1% BSA, unless otherwise stated), 10 mM L-glutamine, 5×10^{-5} M 2-ME, and 1% antibiotic/antimycotic (RPMI-1% FCS or -1% BSA). All experiments reported herein were performed with skin fibroblasts at passages 12–20.

When cultured in RPMI medium containing ≥2.5% FCS, the fibroblasts proliferated constitutively, contained high levels of type α-1 (I) collagen mRNA, and secreted collagen, but when cultured in medium containing ≤1% FCS or 1% BSA, they contained very low levels of type α-1 (I) collagen mRNA and secreted only background levels of collagen (data not shown).

Activation of Cells

Mast Cells. Mast cells were activated via the $Fc_\epsilon RI$ essentially as described previously (18). Briefly, the Cl.MC/C57.1 cells and BMCMC were sensitized in vitro with a monoclonal IgE anti-DNP antibody (H1-DNP-ε-26; reference 24) for 30 min–3 h. The serosal mast cells were sensitized by injecting the mice intraperitoneally with ~7.5 μg of IgE anti-DNP antibody in 1 ml of Hepes-buffered HBSS (Hepes-HBSS). The next day, the mast cells were purified from the peritoneal cavities and further incubated with IgE as noted above for the cultured mast cells. After sensitization, the cells were washed and resuspended at a concentration of 3×10^6 cells/ml in DMEM supplemented with 1% antibiotics/antimycotics, 20 mM L-glutamine and, unless otherwise noted, either 1% FCS, for Cl.MC/C57.1 and BMCMC, or 1% BSA (Pentex [Fraction V, fatty acid-free]; Miles Scientific, Kankakee, IL), for purified serosal mast cells. They were then either stimulated with DNP_{30-40} HSA (50 ng/ml; Sigma Chemical Co.) or left untreated (19). At varying times thereafter, the cell supernatants were harvested and either used fresh or stored at −80°C before assay. Maintenance of the mast cells in DMEM–1% FCS or –1% BSA during the cellular activation period had no adverse effects on their viability, as determined by trypan blue dye exclusion, or on their secretory abilities, as determined by monitoring their release of serotonin and/or TNF-α in response to $Fc_\epsilon RI$ signaling (not shown).

Fibroblasts. For activation of the embryonic skin fibroblasts or 3T3 cells, subconfluent cultures of cells were trypsinized and replated into 8-chamber multi-well microscope slides (Lab-Tek; Miles Scientific) for in situ hybridization, or into 24-well tissue culture plates (Corning Glass Co., Corning, NY) for assays of collagen secretion, and maintained overnight in complete RPMI–10% FCS. Unless otherwise noted, the cells were then washed and incubated in complete RPMI–1% FCS or –1% BSA for an additional ~24 h. Finally, the medium was again replaced with complete RPMI–1% FCS or –1% BSA and then mast cell supernatants, recombinant cytokines, or control medium (vehicle) were added to the cultures, using duplicate sets of wells for in situ hybridization and quadruplicate sets for all other assays. Based on results of preliminary experiments, we used the mast cell supernatants at 10% (final concentration) for in situ hybridization or 50% (final concentration) for assays of collagen secretion.

Molecular Probe for Detection of Type-α-1 (I) Collagen mRNA

A 0.6-kb mouse type α-1 (I) collagen cDNA in the vector pGEM3Z (25) was kindly provided by Dr. L. Van de Water (Beth Israel Hospital and Harvard Medical School, Boston, MA). For in situ hybridization, sense and antisense cRNA probes were prepared by in vitro transcription using ^{35}S-UTP and were purified by affinity elution from glass (RNAid kit; BIO 101, La Jolla, CA).

In Situ Hybridization

Tissues were processed for in situ hybridization essentially as noted (25). Briefly, the tissues were fixed on ice for 3 h in 85% ethanol/5% glacial acetic acid/4% formaldehyde and then processed into 6-μm paraffin sections. Suspensions of purified mouse serosal mast cells were placed on glass microscope slides and allowed to air dry; the cells were fixed for 30 min as above and then transferred into and stored at −20°C in 70% ethanol. The rehydrated slides were digested with proteinase K (1 μg/ml), post-fixed in 4% paraformaldehyde, blocked with acetic anhydride and then hybridized overnight at 50°C with 8.5 ng ^{35}S-cRNA/slide using a hybridization buffer comprising 50% formamide, 5% dextran sulfate, and 10 mM dithiothreitol. The slides were then treated with RNAse A, washed at 65°C in 50% formamide/2× SSC, dehydrated, and dipped in autoradiography emulsion. All slides except those used for the experiment shown in Fig. 4 (which were exposed for 7 d) were exposed for ~3 d; all slides were developed by standard procedures and counterstained in 0.2% toluidine blue.

For analysis of the extent of type α-1 (I) collagen mRNA induction in the embryonic skin fibroblasts, we calculated the mean (+/− SEM) numbers of silver grains/cell in 10–20 randomly chosen cells/replicate, as determined by bright field microscopy using a 100× oil immersion objective (1,000× final magnification). In preliminary experiments, we determined that we could accurately count the number of silver grains/cell only when grain counts were below ~300/cell. Accordingly, we did not attempt to quantify grains/cell in slides, such as those in Fig. 4, with greater levels of autoradiographic signal strength. Each experiment was performed at least twice with each mast cell population tested.

Collagen Assay

We employed the collagen assay of Blumenkrantz and Asboe-Hansen, which is specific for hydroxyproline, but not proline (26). Briefly, secreted proteins were hydrolyzed overnight in 6 N HCl and the amino acids differentially extracted through phase separation across aqueous and organic solvents. Hydroxyproline was quantified spectrophotometrically at OD_{565}, after the addition of *p*-dimethylamino-benzaldehyde to the eluted samples (26). In each assay, HPLC-purified hydroxyproline (Sigma Chemical Co.) was used to generate a standard curve and purified bovine tendon type I collagen (Sigma Chemical Co.) was used as a positive control. We also trypsinized the fibroblasts and counted them in a hemocytometer, and then calculated the levels of secreted hydroxyproline/cell.

The tissue culture media used in these experiments (RPMI– or DMEM–1% FCS) did not contain detectable amounts of hydroxyproline. However, both RPMI 1640 and DMEM contain proline (GIBCO BRL). This result thus confirmed the specificity of the assay for hydroxyproline. The highest levels of hydroxyproline that were detected in supernatants from either Cl.MC/C57.1 mast cells or BMCMC were <5% of those in the supernatants of quiescent embryonic skin fibroblasts and <0.07% of those in the 24 h supernatants of fibroblasts that had been stimulated with activated mast

cell supernatants. Moreover, none of our mast cell populations contained detectable levels of type α-1 (I) collagen mRNA, as determined by Northern blotting (not shown). Mouse mast cells have been reported to produce type IV collagen (27), indicating that production of small amounts of this species of collagen may have accounted for the low levels of hydroxyproline that we detected in the mast cell supernatants.

Biological Assays of Cytokine Activity

TNF-α Assay. TNF-α was detected using an 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazdium bromide (MTT) cytotoxicity assay with L929 cell targets, essentially as reported previously (18). The specificity of the assay for TNF-α was confirmed by use of TNF-α antisera (see above). Internal standards of rTNF-α were used in each assay. The levels of TNF-α in the mast cell supernatants are expressed as U/10^6 cells, where one unit equals the amount of bioactivity required to kill 50% of the cells in the cytotoxicity assay.

TGF-β Assay. A Mv1Lu growth inhibition assay (28) was employed to detect TGF-β bioactivity. Briefly, monolayers of Mv1Lu cells were trypsinized, and then washed and resuspended in DMEM–10% FCS and plated in 96-well plates at 10^4 cells/well. The following day, the medium in quadruplicate wells was replaced with DMEM–1% FCS and serial dilutions of transiently acidified serosal mast cell supernatants. After 48 h of culture, the cell densities in the cultures were then determined by MTT dye uptake, as indicated above for the fibroblast cultures. The TGF-β was activated by addition of 1.5 μl of 6 N HCl/200 μl of supernatant and incubation for 5 min at 22°C, and then the pH was neutralized by the addition of 30 μl of solution of 0.5 M Hepes/0.5 M NaOH (29). The levels of TGF-β in the mast cell supernatants are expressed as U/10^6 cells, where one unit equals that amount of activity required to inhibit by 50% the proliferation of cells in the Mv1Lu growth inhibition assay.

Neutralization of Mast Cell–dependent Fibroblast Responses with Anticytokine Antibodies

To examine the ability of selected anticytokine antibodies to neutralize mast cell–dependent fibroblast responses, we used supernatants from purified serosal mast cells that had been stimulated via the $Fc_\epsilon RI$ for 2 h (19). For each antibody (anti-TNF-α, anti-TGF-β1, and NRS IgGs), we generated specific immunoabsorption matrices by binding the purified IgG to protein A–agarose beads (Affigel Protein A; Bio-Rad Laboratories, Richmond, CA). We incubated 150 μl of a 50% slurry of beads in phosphate-buffered saline with 75 μl of IgG (1 mg/ml) for 2 h at 4°C, then washed the IgG-beads extensively with RPMI–1% BSA. We incubated 150 μl of test serosal mast cell supernatant with 75 μl of IgG-beads for 2 h at 4°C and then centrifuged the mixtures to sediment the beads. The immunoabsorbed supernatants were assayed for residual TNF-α and TGF-β bioactivity and tested for their abilities to induce type α-1 (I) collagen gene expression in embryonic skin fibroblasts.

Induction of PCA Responses

The protocol used for the induction of these $Fc_\epsilon RI$- and mast cell–dependent responses has been reported in detail previously (3, 19). Briefly, we injected ~50 ng of monoclonal IgE anti-DNP antibody in 20 μl of Pipes-HBSS intradermally into the dorsal side of the ears of the mice. After 24 h, the mice were challenged intravenously with 100 μg of $DNP_{30\text{–}40}$ HSA (Sigma Chemical Co.) in 200 μl of Hepes-HBSS containing 0.5% Evan's blue dye. The presence of PCA reactivity was confirmed by assessing the extravasation of Evan's blue dye (3, 19). At varying times after antigen challenge, ear biopsies were processed for in situ hybridization.

Mast Cell Reconstitution of Genetically Mast Cell–deficient $WBB6F_1$-W/W^v Mice

We have reported previously that $WBB6F_1$-W/W^v mice can be selectively and locally repaired of their mast cell deficiency by the intradermal injection of BMCMC derived from $WBB6F_1$-+/+ mice. These mast cells fully reconstitute the ability of the recipients to express mast cell–dependent PCA responses (3, 23). Briefly, bone marrow cells from $WBB6F_1$-+/+ mice were maintained in culture with an exogenous source of IL-3 for ~3 wk, after which the cells remaining in culture were composed of ≥98% immature mast cells (BMCMC). 5×10^5 BMCMC in 20 μl of DMEM were injected intradermally into the dorsal side of the left ear of each W/W^v mouse (3). The mice were used 10 wk later, a period sufficient to permit maturation of the BMCMC that had been injected into the ear skin (3).

Statistical Analyses

Statistical analyses were performed using the program Statview 512+ (Abacus Concepts, Berkeley, CA). The data were subjected to analysis of variance (ANOVA) testing to determine the overall impact of sample treatments within an experiment, with additional post-hoc testing using the Fisher Protected Least Significant Difference (PLSD) test to determine the statistical significance of individual sample treatments on the parameters in question. Results are reported as significant only if both the ANOVA and Fisher PLSD tests yielded a probability (p) value of ≤ 0.05. Correlation analyses were also performed with the Statview 512+ package. All data are reported as mean ± SEM.

Results

Increased Levels of Type I Collagen mRNA Occur in IgE- and Mast Cell-dependent PCA Reactions In Vivo. We first assessed whether IgE-dependent mast cell activation was associated with augmentation of collagen gene expression in vivo, by using in situ hybridization to search for type α-1 (I) collagen mRNA expression during PCA responses in the ears of BALB/c mice (Fig. 1). The reaction sites could be easily recognized histologically by the presence of degranulated mast cells, tissue edema, and, beginning ~4 h after antigen challenge, neutrophil and later monocyte infiltration. However, control tissues injected with medium rather than IgE showed no signs of mast cell degranulation or interstitial edema, nor did they specifically bind the antisense probe (Fig. 1, *A* and *B*). On the other hand, type α-1 (I) collagen mRNA was induced in some cells at the PCA reaction sites by 60 min (Fig. 1 *C*). By 2 h, before inflammatory cells had been recruited to the reaction sites, many cells in the PCA sites, as well as occasional cells more distant from the reactions, strongly hybridized with the antisense probes (Fig. 1 *D*). These results indicate that the collagen mRNA-positive cells were indeed resident cells of the dermis and that the initial upregulation of collagen mRNA levels did not depend on the recruitment of circulating inflammatory cells. Thereafter, both the numbers of cells that bound the antisense probe and the intensity of the mRNA signal in each cell increased dramatically until

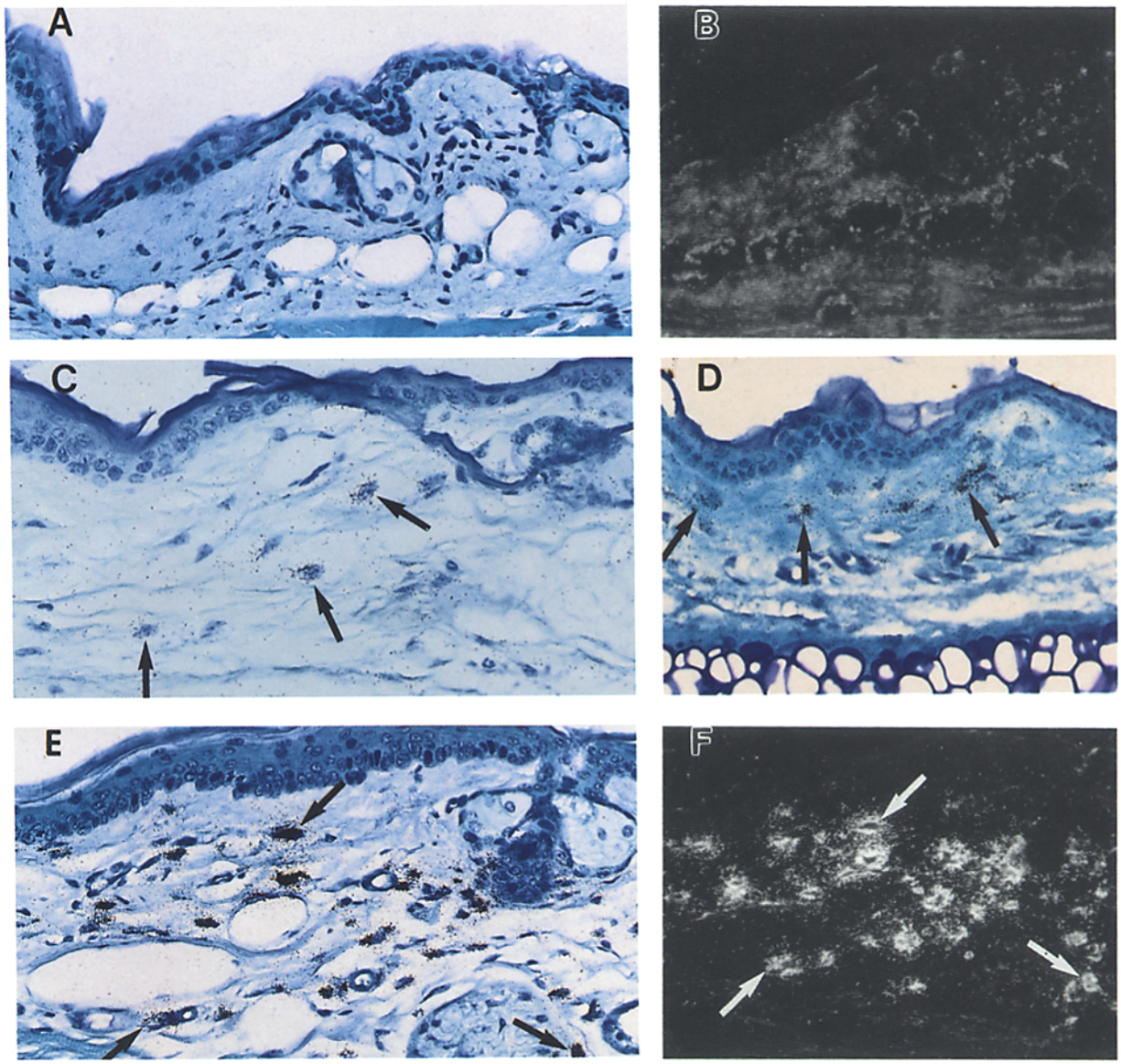

Figure 1. In situ hybridization analysis of type α-1 (I) collagen mRNA induction in vivo during PCA responses in the skin of mice. The ears of BALB/c mice were injected intradermally with IgE anti-DNP antibodies or antibody diluent and, 1 d later, the mice were challenged intravenously with DNP_{30-40} HSA. (*A*) Light field or (*B*) dark field photomicrographs of the same area of control (diluent injected) sites 16 h after antigen challenge. Little or no hybridization with the ^{35}S-labeled antisense probes is evident. (*C* and *D*). Light field photomicrographs of PCA reaction sites 1 h (*C*) or 2 h (*D*) after antigen challenge. Several cells (some indicated with *arrows*) are positive for type α-1 (I) collagen mRNA. (*E*) Light field or (*F*) dark field photomicrographs of the same area of a PCA reaction site 16 h after antigen challenge. Many cells (three of which are indicated with arrows in both *E* and *F*) exhibit strong hybridization signals with the ^{35}S-labeled antisense probe for type α-1 (I) collagen mRNA. All bright field photomicrographs × 580; dark field photomicrographs ×420. Toluidine blue counterstain. All autoradiographic exposures were for 3 d.

~16 h (Fig. 1, *E* and *F*) to 24 h post challenge. The intensity of the signals then began to wane until, by 48 h after antigen challenge, labeling was back to baseline levels (not shown).

To demonstrate unequivocally that mast cells were required for the development of the increased levels of type α-1 (I) collagen mRNA that appeared at IgE-dependent PCA reactions, we performed experiments in genetically mast cell–deficient $WBB6F_1$-W/W^v (W/W^v) mice, the congenic +/+ mice, and W/W^v mice that had been locally and selectively repaired of their mast cell deficiency.

The kinetics and extent of type α-1 (I) collagen mRNA expression at PCA reaction sites in $WBB6F_1$-+/+ mice were very similar to those in BALB/c mice (Fig. 2, *A* and *B*). By contrast, when genetically mast cell–deficient W/W^v mice were treated with IgE and antigen in an identical fashion, we observed no detectable expression of type α-1 (I) collagen mRNA in the tissues (Fig. 2, *C* and *D*). However, when

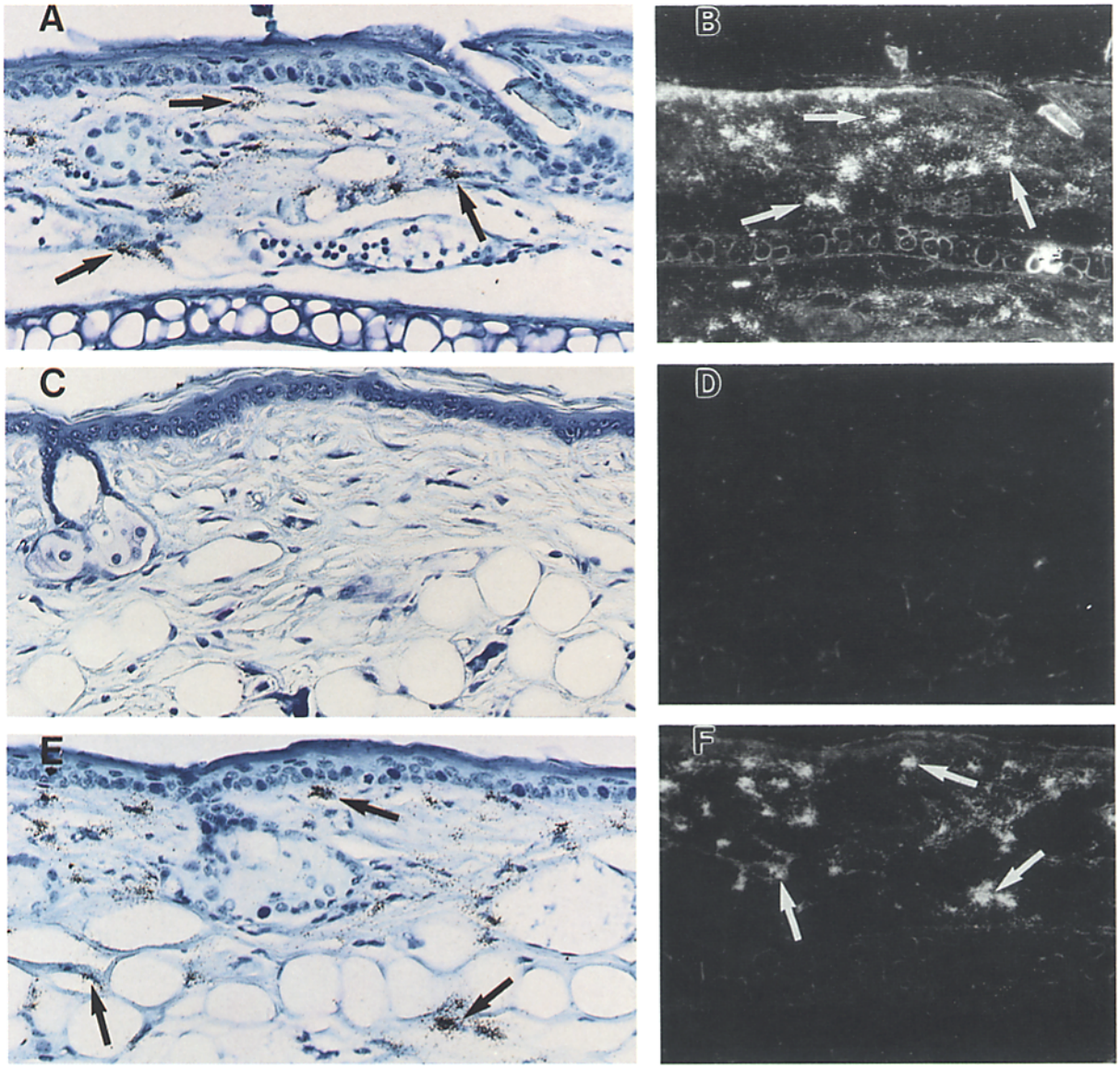

Figure 2. Mast cell dependence of the expression of type α-1 (I) collagen mRNA at PCA reaction sites. Light field (*A*, *C*, and *E*) or dark field (*B*, *D*, and *F*) photomicrographs of IgE-injected ears of normal $WBB6F_1$-+/+ mice, (*A* and *B*), mast cell–deficient $WBB6F_1$-*W/W^v* mice (*C* and *D*), or mast cell–reconstituted $WBB6F_1$-*W/W^v* mice (*E* and *F*) 16 h after intravenous antigen challenge. The pairs of light or dark field photomicrographs are from the same field of sections hybridized to ^{35}S-labeled antisense probes for type α-1 (I) collagen mRNA and then counterstained with toluidine blue. Many type α-1 (I) collagen mRNA positive cells (some of them indicated with *arrows*) are detectable in the $WBB6F_1$-+/+ mice (*A* and *B*) or mast cell-reconstituted $WBB6F_1$-*W/W^v* mice (*E* and *F*), but none are apparent in the tissues of the mast cell–deficient $WBB6F_1$-*W/W^v* mice (*C* and *D*). All bright field photomicrographs × 580; dark field photographs × 320. All autoradiographic exposures were for 3 d.

$WBB6F_1$-*W/W^v* mice were tested 10 wk after the selective repair of their cutaneous mast cell deficiency, PCA reactions could be elicited, as previously reported (3, 23), and these reactions were associated with the high level induction of type α-1 (I) collagen mRNA expression (Fig. 2, *E* and *F*).

Taken together, the results of our in vivo experiments demonstrated that high level induction of type α-1 (I) collagen gene expression occurred in skin which was challenged with IgE and specific antigen, that this change, at least at the earliest stages of the response, did not reflect the infiltration of inflammatory cells into the tissues, and that the development of the response was entirely dependent on mast cells.

Mast Cells Activated Via the $Fc_\epsilon RI$ Induce Type α-1 (I) Collagen mRNA Accumulation in Fibroblasts In Vitro. We used in vitro approaches to investigate possible mechanisms by which IgE-dependent mast cell activation might lead to increased expression of type α-1 (I) collagen. We first assessed whether supernatants from cloned Cl.MC/C57.1 mast cells could induce changes in steady state levels of collagen mRNA in skin fibroblasts. Since FCS itself can be mitogenic for fibro-

blasts, we also examined the effects in this system of increasing concentrations of FCS in the culture medium during both mast cell activation and subsequent incubation of fibroblasts with mast cell supernatants (Fig. 3 *A*). We used ^{35}S-labeled sense (negative control) and antisense type α-1 (I) collagen cRNAs to probe the fibroblasts after the addition of the supernatants, and assessed the specific collagen mRNA levels by counting the numbers of silver grains deposited over individual fibroblasts in these preparations. In all of our experiments, fibroblasts incubated with ^{35}S-sense cRNA probes were uniformly negative (i.e., ≤0.1 ± 0.01 silver grain/cell). By contrast, fibroblasts incubated for 16 h with medium lacking FCS or mast cell supernatants gave a signal with ^{35}S-antisense probes, reflecting "baseline" levels of collagen mRNA, of 44 ± 5 silver grains/cell (Fig. 3 *A*).

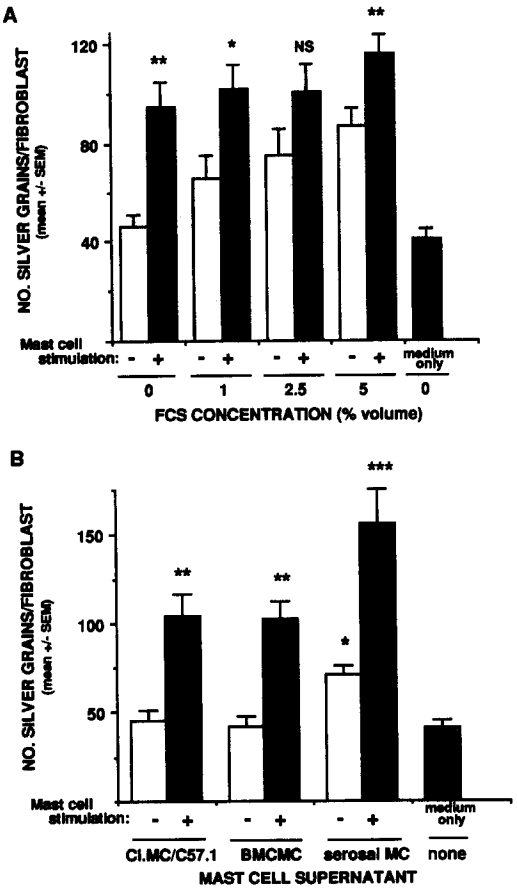

Figure 3. Type α-1 (I) collagen mRNA levels, expressed as the number of silver grains/fibroblast by in situ hybridization with a ^{35}S-labeled antisense cRNA probe, in skin fibroblasts stimulated for 16 h with supernatants of activated or quiescent mouse mast cells. The supernatants were from mast cells that had been sensitized with a monoclonal IgE anti-DNP antibody and then either challenged with DNP_{30-40} HSA (+) or left unchallenged (−) for 2 h. (*A*) Effect of FCS concentration on the induction of type α-1 (I) collagen in fibroblasts incubated with Cl.MC/C57.1 mast cell supernatants. *, $p < 0.05$; **, $p < 0.01$; NS = no significant difference; vs. values in fibroblasts incubated with supernatants of unstimulated mast cells at the same FCS concentration. (*B*) Comparison of the abilities of supernatants from unstimulated (−) or activated (+) Cl.MC/C57.1 mast cells, BMCMC, or freshly purified serosal mast cells (>99% purity) to stimulate type α-1 (I) collagen mRNA expression in skin fibroblasts. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. medium alone (n = 10/condition).

In the complete absence of FCS, supernatants from 2 h cultures of unstimulated Cl.MC/C57.1 cells had no significant effect above background on the expression of collagen mRNA by the fibroblasts, whereas medium from parallel cultures of mast cells activated via the $Fc_\epsilon RI$-induced levels of signal for collagen mRNA expression which were more than twice baseline ($p \leq 0.01$; Fig. 3 *A*). As expected, FCS itself (but not 1% BSA, not shown) significantly increased collagen mRNA signal strength in fibroblasts incubated with supernatants from unstimulated mast cells ($F_{[8, 81]} = 8.25$; $p \leq 0.001$) (Fig. 3 *A*). Indeed, there was a strong correlation between the concentration of FCS in the cultures and the steady state levels of fibroblast collagen mRNA which were detected by in situ hybridization, whether we added supernatants from unstimulated Cl.MC/C57.1 cells (Fig. 3 *A*; $r^2 = 0.83$, $n = 4$) or medium alone (not shown). By contrast, in fibroblast that were incubated with supernatants from activated mast cells, the concentrations of FCS employed in the cultures did not significantly affect the induction of collagen mRNA ($F_{[3, 36]} = 0.791$, $p = 0.51$). At 0, 1, and 5% FCS, supernatants from activated Cl.MC/C57.1 cells induced higher level expression of collagen mRNA than did the supernatants from unstimulated mast cells (Fig. 3 *A*).

We next compared the abilities of growth factor–independent Cl.MC/C57.1 mast cells, growth factor–dependent mouse BMCMC, and freshly purified mouse serosal mast cells to induce type 1 collagen responses in these fibroblasts (Fig. 3 *B*). In these and all subsequent experiments, fibroblasts were maintained in the same medium used for mast cell activation: 1% FCS for Cl.MC/C57.1 cells or BMCMC, 1% BSA for serosal mast cells. Supernatants from all three populations of activated mast cells induced markedly elevated steady state levels of fibroblast collagen mRNA ($F_{[3, 40]} = 15.34$, $p \leq 0.0001$; Fig. 3 *B*), with increases in grain count values compared to those in cells incubated in medium alone of 157% for Cl.MC/C57.1 cells, 150% for BMCMC, and 281% for serosal mast cells (Fig. 3 *B*). By contrast, supernatants from unstimulated Cl.MC/C57.1 cells or BMCMC had no significant effect above background (medium alone). While there were no statistically significant differences between the collagen mRNA signals in fibroblasts treated with supernatants of unstimulated Cl.MC/C57.1 cells, BMCMC or serosal mast cells ($p \geq 0.05$, $n = 10$), values in fibroblasts incubated with the unstimulated serosal mast cell supernatants in this experiment were slightly but significally elevated when compared with those in cells incubated in medium alone ($p = 0.048$, $n = 10$).

We also investigated the kinetics of mast cell activation-dependent type α-1 (I) collagen mRNA accumulation in fibroblasts (Fig. 4). As noted above, even with prolonged (7 d) autoradiographic exposure times, sense cRNA probes did not bind to the fibroblasts, even after stimulation with supernatants from activated BMCMC (Fig. 4 *A*), and unstimulated fibroblasts (Fig. 4 *B*), or those incubated with supernatants from unstimulated BMCMC (not shown), bound only low levels of the ^{35}S-antisense probes. Supernatants from acti-

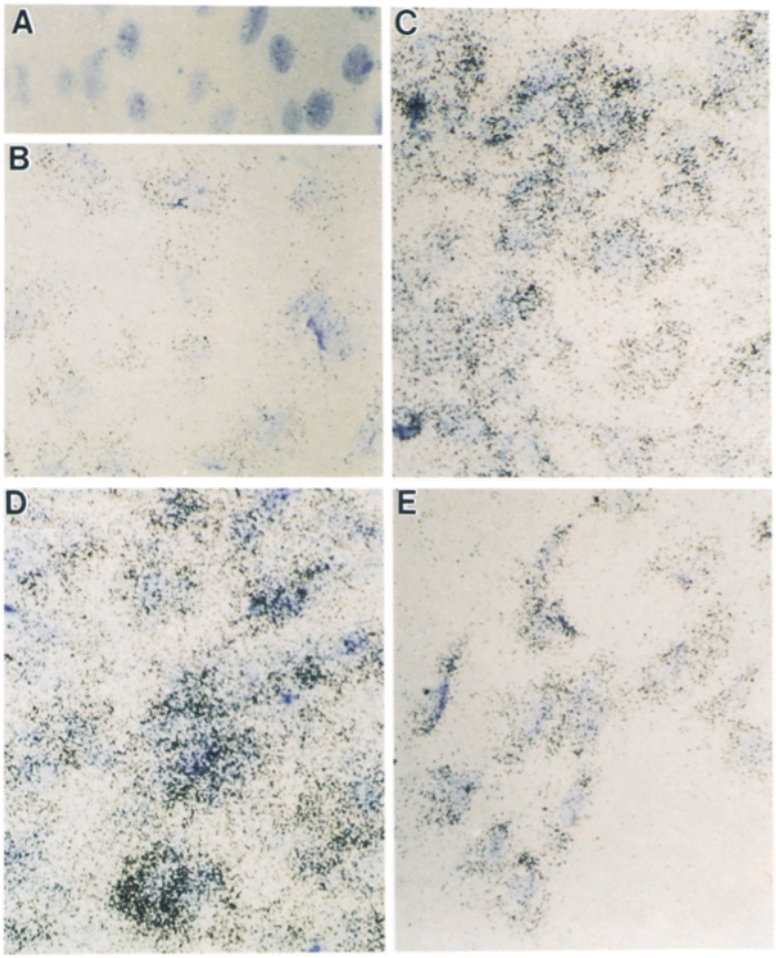

Figure 4. Kinetics of induction of type α-1 (I) collagen mRNA expression in skin fibroblasts incubated with supernatants from unstimulated (*B*) or activated (*A*, *C–E*) BMCMC. (*A*) Fibroblasts stimulated for 90 min with activated mast cell supernatants and probed with the ^{35}S-sense (negative control) cRNA. (*B*) Unstimulated fibroblasts probed with the ^{35}S-antisense cRNA. (*C–E*) Fibroblasts stimulated with 2 h supernatants from activated BMCMC for (*C*) 90 min, (*D*) 8 h, or (*E*) 24 h and then hybridized with the ^{35}S-antisense cRNA probe. All autoradiographic exposures were for 7 d.

vated BMCMC induced a high level of expression of collagen mRNA in the fibroblasts (Fig. 4, *C–E*). The signal strength was noticeably elevated 90 min after addition of the supernatants of activated BMCMC (Fig. 4 *C*), was markedly increased by 8 h (Fig. 4 *D*), appeared to have waned somewhat by 24 h (Fig. 4 *E*), and was reduced further by 48 h (not shown).

Mast Cell Activation Promotes Collagen Secretion by Fibroblasts. To confirm that augmented collagen secretion occurred in the transcriptionally active populations of mast cell–stimulated fibroblasts, we measured the collagen production by these cells, as determined by the levels of hydroxyproline in the culture medium (Fig. 5). We found that supernatants from activated, but not unstimulated, Cl.MC/C57.1 cells significantly increased fibroblast collagen secretion at 16 or 32 h after addition of the supernatants ($F_{[8,\ 13]} = 4.27$, $p \leqslant 0.01$). Additional experiments showed that supernatants from quiescent or activated BMCMC had effects on collagen secretion by 3T3 fibroblasts that were virtually identical to those observed in primary cultures of embryonic skin fibroblasts which had been stimulated with Cl.MC/C57.1 mast cell supernatants ($r^2 = 0.988$, $n = 6$; not shown).

Role of TGF-β and TNF-α in Mast Cell–mediated Induction of Fibroblast Type 1 Collagen Expression. We employed normal rabbit serum IgG, anti-TGF-β1 IgG or anti-TNF-α IgG matrices to absorb supernatants of serosal mast cells that had been activated via the $Fc_\epsilon RI$, and then tested the absorbed supernatants for their abilities to induce type α-1 (I) collagen mRNA expression in embryonic skin fibroblasts (Fig. 6). Supernatants from unstimulated serosal mast cells cultured for 2 h in 1% BSA medium contained low levels of TNF-α (0.73 ± 1.4 U/10^6 cell equivalents) and TGF-β (1.1 ± 0.3 U/10^6 cells) bioactivities. Absorption of the supernatants from activated mast cells with the NRS IgG-agarose beads had no significant affect on the levels of TGF-β or TNF-α. Thus the unabsorbed activated mast cell supernatants contained 169 ± 11 U TNF/10^6 cell equivalents and 9.7 ± 1.4 U TGF-β/10^6 cell equivalents while the NRS IgG matrix-absorbed supernatants contained 155 ± 20 U TNF/10^6 cell equivalents and 12.1 ± 1.2 U TGF-β/10^6 cell equivalents (both, $p > 0.05$, $n = 6$). The anti-TNF-α absorptions removed 94 ± 2% of the TNF-α activity from the supernatants while the anti-TGF-β1-agarose beads removed 97 ± 6% of the TGFβ bioactivity. The anti-TNF-α matrices had no effect on the TGF-β activities of the activated cell supernatants, while the anti-TGF-β1 matrices slightly reduced (by 9 ± 3%) the TNF-α bioactivity.

In this set of experiments, supernatants of unstimulated serosal mast cells cultured for 2 h in medium alone had no

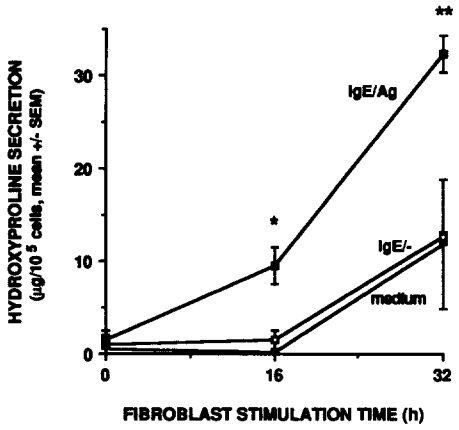

Figure 5. Effect of supernatants from unstimulated (IgE/−) or activated (IgE/Ag) Cl.MC/C57.1 mast cells, or medium alone, on the secretion of collagen (hydroxyproline) by subconfluent monolayers of embryonic skin fibroblasts 16 or 32 h after adding mast cell supernatants, the number of fibroblasts in each culture was determined by direct counting of the trypsinized cells, and the amount of collagen secreted was assessed by measuring the level of hydroxyproline in the culture supernatants. *, $p < 0.05$; **, $p < 0.01$ vs. value for IgE/− or medium alone.

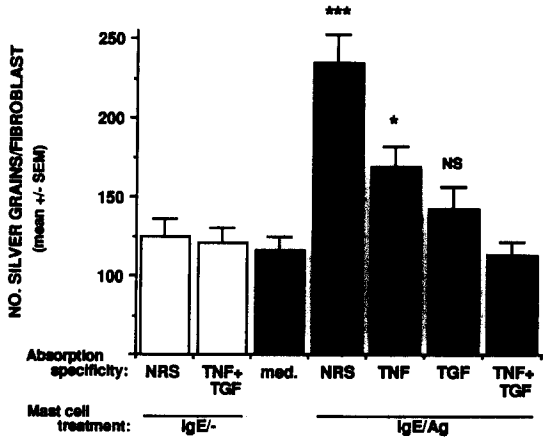

Figure 6. Role of TGF-β and TNF-α in the mast cell activation–dependent induction of type α-1 (I) collagen mRNA in mouse skin fibroblasts in vitro. Supernatants from purified serosal mast cells that were sensitized with IgE and then either were left unstimulated (IgE/−) or were challenged with specific antigen (IgE/Ag) were absorbed with NRS, anti-TGF-β1, anti-TNF-α, or a combination of anti-TGF-β1/anti-TNF-α IgG immunoaffinity matrices, and then tested by in situ hybridization with specific antisense cRNA probes for their residual abilities to induce type α-1 (I) collagen mRNA expression in embryonic skin fibroblasts, as in Fig. 4. *, $p < 0.05$; ***, $p < 0.001$; NS, not significant; vs. medium alone (n = 10, condition).

statistically significant effect on fibroblast type α-1 (I) collagen mRNA expression, as assessed by grain count analysis of in situ hybridization preparations (Fig. 6; $p > 0.05$ versus medium alone, n = 10). As noted previously, supernatants from activated serosal mast cells dramatically upregulated fibroblast collagen expression (Fig. 6), with statistically indistinguishable effects observed with unabsorbed supernatants (165% increase in grains/cell vs. medium alone, $p < 0.001$, n = 10) or NRS IgG-absorbed supernatants (173% increase vs. medium alone, $p < 0.001$, n = 10). By contrast, the anti-TNF-α or anti-TGF-β1 matrices depleted the activated serosal mast cell supernatants of 59 ± 8% or 77 ± 7%, respectively, of their ability to augment signal for type α-1 (I) collagen mRNA. Absorption with anti-TGF-β1 matrices was more effective than absorption with anti-TNF-α, in that values for fibroblasts incubated with medium alone or with anti-TGF-β1-absorbed mast cell supernatants were statistically indistinguishable, whereas the anti-TNF-α–absorbed supernatants had a residual activity that was still significantly greater (by ~74%) than that of medium alone ($p \leq 0.05$, n = 10). Absorption with both anti-TGF-β1 and anti-TNF-α matrices was significantly more effective than absorption with either the anti-TNF-α ($p \leq 0.001$, n = 10) or the anti-TGF-β1 ($p \leq 0.05$, n = 10) matrices alone.

Discussion

Our results show that the $Fc_\epsilon RI$-dependent activation of mouse mast cells can induce increased levels of type α-1 (I) collagen gene expression in mouse skin fibroblasts in vitro or in vivo. Analysis of dermal fibroblast collagen gene expression by in situ hybridization demonstrated a striking increase in collagen mRNA at PCA reaction sites in normal BALB/c or $WBB6F_1$-+/+ mice and in genetically mast cell–deficient $WBB6F_1$-W/W^v mice that had been selectively repaired of their cutaneous mast cell deficiency, but not in $WBB6F_1$-W/W^v mice that remained mast cell deficient. These results demonstrate unequivocally that the increased expression of type α-1 (I) collagen mRNA which developed at sites of PCA reactions was mast cell dependent.

In normal mice, increased signals for type α-1 (I) collagen mRNA were noted at PCA reaction sites 1 h after administration of specific antigen and were further increased by 2 h after challenge, before the recruitment of significant numbers of circulating leukocytes to the sites (3). This finding indicates that the increased levels of dermal collagen mRNA expression that are observed at the early stages of these IgE- and mast cell–dependent reactions can occur independently of the action of recruited leukocytes. However, this finding can not rule out the possibility that activated mast cells promote dermal fibroblast collagen gene expression in vivo at least in part by influencing the function of a third cell type resident in the skin.

We therefore used in vitro approaches to investigate mechanisms that might account for the ability of activated mast cells to promote fibroblast collagen mRNA expression. We found that the $Fc_\epsilon RI$-dependent activation of all three mouse mast cell populations tested resulted in the release of mediators that markedly increased type α-1 (I) collagen mRNA expression in mouse embryonic skin fibroblasts in vitro (Fig. 3 *B*). These mast cell populations included a cloned, growth factor–independent cell line (Cl.MC/C57.1), as well as primary cultures of immature, IL-3–derived BMCMC and freshly isolated mature serosal mast cells, both of which contained >98% mast cells. These experiments thus strongly indicate

that mouse mast cell activation can directly augment fibroblast collagen mRNA expression.

We (1, 17) and others (6) have proposed that mast cells might influence fibroblast gene expression and protein synthesis through the production of multifunctional cytokines. Our results provide the first direct support for this hypothesis. We found that absorption of the supernatants of activated serosal mast cells with antibodies to TGF-β1, which depleted the TGF-β bioactivity in these supernatants by 97 $\pm$ 6%, also markedly reduced (by $\sim$78 $\pm$ 7%, according to autoradiographic grain count analysis) the ability of these supernatants to augment fibroblast type α-1 (I) collagen mRNA levels in vitro. Absorption of the same supernatants with antibodies to TNF-α also reduced their ability to augment signal for fibroblast collagen mRNA, but to a significantly lesser extent than did absorption with antibodies to TGF-β1, and the combination of antibodies to both cytokines was significantly more effective than either one alone. Based on these findings, we propose that both TGF-β1 and TNF-α represent mediators that can contribute to the ability of activated mast cells to augment fibroblast collagen mRNA levels.

A comparison of the findings of our in vivo and in vitro in situ hybridization experiments suggests that the increases in fibroblast type α-1 (I) collagen mRNA levels which occurred at sites of IgE-dependent mast cell activation in vivo may have been even greater than those detected when cultured fibroblasts were stimulated with the supernatants of mast cells that had been activated via the $Fc_\epsilon RI$ in vitro. These findings might have reflected differences in the phenotypes of the mast cell (and/or fibroblast) populations that were analyzed in vivo as opposed to in vitro, or the influence of additional cell types that were present in vivo but not in vitro. However, the precise relationship between the numbers of silver grains/cell which are detected by in situ hybridization, and the actual mRNA copy numbers/cell, is unknown. Nor is it certain that this relationship necessarily is the same for slides derived from in vivo as opposed to in vitro experiments. Accordingly, autoradiographic grain count analysis is best regarded as a "semiquantitative" assessment of the levels of mRNA for the transcript of interest.

Several lines of evidence indicate that the increases in the steady-state levels of fibroblast collagen mRNA, or augmented collagen synthesis, which are induced by a single instance of IgE-dependent mast cell activation are rather transient. Our in vivo studies showed that the increase in type α-1 (I) collagen mRNA that occurred at PCA reaction sites peaked at $\sim$16–24 h after antigen challenge and waned to background levels by 48 h. Notably, the kinetics of this response paralleled very closely the kinetics of the mast cell–dependent granulocyte infiltration that we observed in these reactions (3). The addition of supernatants of activated mast cells to skin fibroblasts in vitro also produced a transient increase in steady-state levels of fibroblast type α-1 (I) collagen mRNA (Fig. 4), and the kinetics of this response was very similar to that observed in dermal fibroblasts at PCA reaction sites in vivo. Finally, we measured the hydroxyproline content of mouse skin at various intervals after the induction of an IgE-dependent PCA reaction. At 12–15 h after antigen challenge, we found only a slight increase (of $\sim$26%) in hydroxyproline levels compared to those at control reaction sites not injected with IgE. Moreover, these increases achieved statistical significance in only one of three experiments.

Our findings are consistent with observations indicating that the development of clinically or histologically significant tissue fibrosis is a feature of chronic allergic responses, such as long-term allergic asthma or atopic dermatitis, rather than isolated type I hypersensitivity responses (1, 6, 7). And even though we have shown that a single instance of mast cell activation can rapidly induce changes in local levels of dermal collagen gene expression, leukocytes recruited to sites of mast cell activation in vivo may also importantly contribute to the regulation of collagen production in these tissues. Accordingly, it will be of interest to develop model systems that permit evaluation of the effects of repeated or persistent mast cell activation on tissue levels of collagen and other components of the extracellular matrix, and also to determine the extent to which any changes in the extracellular matrix that are observed in these responses reflect the direct or indirect effects of mast cell activation.

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