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Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion

(tumor necrosis factor/skin/organ culture/inflammation/cytokines)

LYNN M. KLEIN, ROBERT M. LAVKER, WENDY L. MATIS, AND GEORGE F. MURPHY

Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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ABSTRACT **To understand better the role of mast cell secretory products in the genesis of inflammation, a system was developed for *in vitro* degranulation of human mast cells in skin organ cultures. Within 2 hr after morphine sulfate-induced degranulation, endothelial cells lining microvessels adjacent to affected mast cells expressed an activation antigen important for endothelial–leukocyte adhesion. Identical results were obtained when other mast cell secretagogues (anti-IgE, compound 48/80, and calcium ionophore A23187) were used. Induction of this antigen was abrogated by preincubation with cromolyn sodium, an inhibitor of mast cell secretion, and by antiserum to tumor necrosis factor α. These findings indicate that degranulation of mast cells activates dermal endothelium through tumor necrosis factor-dependent mechanisms. This event may be critical to the elicitation phase of cutaneous inflammation.**

Mast cells are resident, granule-containing secretory cells situated about blood vessels of host–environmental interfaces, such as skin and mucosae. Identified more than a century ago by Ehrlich (1), it has been hypothesized that mast cells play a central role in the genesis and modulation of cellular inflammatory responses (2–4). For example, delayed-type hypersensitivity reactions (DHRs) in mice are elicited preferentially at cutaneous sites enriched in mast cells, such as ear and footpad (5). Moreover, pharmacologic inhibition of release of mast cell secretory products with the cromolyn-like drug proxicromil inhibits the DHR (6). Similarly, in sensitized guinea pigs, the DHR is abrogated by first purging mast cells at the challenge site with a local injection of the mast cell secretagogue compound 48/80 (7). Furthermore, mice that are genetically deficient in mast cells (W/W^v and $Sl/Sl/^d$) have been reported (4) to exhibit diminished DHR. An unresolved issue in all such studies is whether mast cell degranulation products are capable of primarily triggering leukocytic infiltration of the skin or whether mast cells degranulate secondarily as a result of the inflammatory cascade (8, 9).

Upon degranulation mast cells release histamine, resulting in gaps between adjacent endothelial cells. This enhances vascular permeability for macromolecules within serum (10–12), although it does not always result in inflammatory cell migration into the perivascular compartment (8). Mast cells also contain heparin, a physiological anticoagulant that influences angiogenesis (13). Neither of these observations, however, provides insight as to how mast cells may be involved in the initial accumulation of inflammatory cells in injured or antigen-stimulated tissue. Such accumulation requires adhesion of circulating leukocytes to stimulated or "activated" endothelial cells lining blood vessels, transit of leukocytes across the vascular wall, and directional migration of these cells once liberated in the perivascular space.

Recent studies suggest (14) that endothelial activation and associated adhesion of blood leukocytes to endothelial surfaces are mediated by specific glycoproteins inducible on human vascular endothelial cells. One such glycoprotein [endothelial–leukocyte adhesion molecule 1 (ELAM-1), ref. 15] has been cloned, sequenced, and is demonstrable by the monoclonal antibody H4/18 (16) exclusively on cytokine-stimulated or inflamed vascular endothelium. ELAM-1 and related molecules correlate with endothelial activation and with endothelial-dependent mechanisms of neutrophil and mononuclear cell adhesion to the endothelial cell surface *in vitro* (17, 18). In organ cultures of human skin, ELAM-1 may be induced only in endothelium of post-capillary venules (19), the targets of most cutaneous inflammatory reactions. Moreover, expression of ELAM-1 is integral to the evolution of the cutaneous delayed hypersensitivity response (20). Undefined factors released by skin explants into culture supernatants are capable of inducing ELAM-1 in target monolayers of large-vessel endothelial cells (10). The recombinant immunoregulatory proteins (cytokines) tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) elicit endothelial activation and expression of ELAM-1 *in vitro* (21–23). In the skin, IL-1 is produced by epithelial cells, and both IL-1 and TNF-α are produced by monocytes (24). Although it is likely that these and other endogenous cytokines are responsible for induction of ELAM-1 *in vivo*, the specific stimuli and cells responsible for their release have not been defined.

To understand the cellular events associated with endogenous mediator release that triggers endothelial activation and endothelial–leukocyte adhesion, we have investigated (25) the sequential ultrastructural and immunophenotypic morphology of the cutaneous DHR in normal human skin. Degranulation of mast cells about superficial vessels was the first ultrastructural alteration noted, 4 hr after contact antigen (dinitrochlorobenzene) challenge. Degranulation anteceded accumulation of inflammatory cells (predominantly T lymphocytes) by ≈20 hr. We, therefore, reasoned that endogenous mediators crucial for local recruitment of circulating leukocytes may be liberated as a result of degranulation. In this report, we demonstrate that mast cell degranulation in the perivascular space results in rapid, cytokine-dependent induction of the endothelial-activation antigen ELAM-1 in adjacent microvasculature. Moreover, this induction appears to be dependent upon endogenous release of a substance with immunologic properties of TNF-α. These findings establish mast cell secretion as a critical link in the development of inflammatory responses.

MATERIALS AND METHODS

Organ Culture. Normal neonatal human foreskins were obtained from elective surgical circumcision procedures. In

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Abbreviations: ELAM-1, endothelial–leukocyte adhesion molecule 1; TNF-α, tumor necrosis factor α; IL-1, interleukin 1; MS, morphine sulfate; DHR, delayed-type hypersensitivity reaction.

certain experiments, normal adult human skin obtained as 4-mm punch biopsies from the upper arm was also cultured and evaluated (see below) to ensure that findings were not peculiar to the neonate. All procedures were performed under sterile conditions. Specimens were immediately immersed in RPMI 1640 medium (Flow Laboratories, McLean, VA) to which 10% (vol/vol) fetal calf serum, 1% penicillin/streptomycin, and 10 mM L-glutamine (GIBCO) had been added. While suspended, foreskin was cut into 1- to 2-mm pieces and placed in 35-mm cell wells, epidermal surface up. Sufficient medium (≈1000 μl) to cover 80% of the explant surface area (epidermis uncovered) was added (complete submersion results in disordered epidermal maturation and enhanced epithelial outgrowth). Explants were incubated at 37°C with humidified 5% CO_2/95% air. Addition of polymyxin B sulfate (50 μg/ml), which neutralizes endotoxins, to this culture system does not influence ELAM-1 induction, and medium alone does not elicit ELAM-1 induction in cultured monolayers of human umbilical vein endothelium (19). Explant viability in this model has been repeatedly assessed by ultrastructure, the ability to induce class II histocompatibility antigens on the surface of target cell types (e.g., endothelial cells) with recombinant interferon γ (26), and the demonstration of continued constitutive endothelial reactivity for anti-factor VIII antibody (19). In each culture, the medium was supplemented with the indicated mast cell secretagogues, drugs, or antisera. Explants were harvested every 2 hr for 6 hr and at 24 hr and 48 hr, as indicated.

Mast Cell Secretagogues. The mast cell secretagogues consisted of morphine sulfate (MS; Wyeth), compound 48/80 (Burroughs Wellcome), calcium ionophore A23187 (Sigma), and rabbit anti-human IgE (IgG fraction) (Dako, Carpinteria, CA). Final concentrations after addition to culture medium consisted of MS at 10, 100, 1000, and 5000 μmol/liter; compound 48/80 at 0.01, 0.1, 0.2, 1, and 10 mg/ml; calcium ionophore at 5, 10, 50, and 100 μg/ml; and anti-IgE at 0.0078, 0.078, and 0.78 mg/ml. Intervals of exposure for all experiments, except those using anti-IgE, were 30, 45, 60, and 120 min, and immediately after removal of explants from secretagogue-containing medium, tissue was processed for ultrastructural examination. Optimal concentrations were determined by ultrastructural analysis to define maximum mast cell degranulation in the absence of alterations in cells or their organelles indicative of nonspecific injury. In experiments using anti-IgE, foreskin explants were first incubated overnight at 37°C in 30% (vol/vol) human atopic serum, rinsed thoroughly, and then exposed to anti-IgE for 45 min. Adequacy of degranulation, as compared to controls [replicate cultures exposed to atopic serum followed by a 45-min incubation in rabbit IgG (0.79 mg/ml; Jackson ImmunoResearch)], was assessed morphologically. In certain experiments, skin explants were preincubated for 1 hr in the mast cell inhibitor cromolyn sodium (Sigma) at 10, 50, 100, 500, and 1000 μg/ml in culture medium prior to addition of secretagogue.

Antibodies and Mediators. The monoclonal antibody H4/18 (courtesy of M. A. Gimbrone, Jr., and Jordan Pober, Harvard Medical School, Boston) was used for immunohistochemical detection of ELAM-1 epitopes in frozen tissue sections. This antibody was developed by immunizing BALB/c mice with cultured umbilical vein endothelial cell harvests (16). It is of the IgG1(κ) isotype, and irrelevant isotype-specific controls (e.g., K16/16) do not demonstrate crossreactivity with human foreskin endothelium using these techniques. H4/18 was used in the form of a reconstituted ammonium sulfate precipitate from ascites fluid diluted 1:500. Polyclonal antibodies used in blocking experiments consisted of rabbit anti-human TNF-α (Genzyme) and rabbit anti-human IL-1α and β (Genzyme). Explants were incubated in anti-TNF-α at 500, 5000, and 50,000 units/ml and in anti-IL-1 at 50, 100, and 200 units/ml. Replicate explants were also incubated in identical concentrations of rabbit serum to exclude nonspecific interference with mast cell mediator effects, and ultrastructure was performed to preclude the possibility that antiserum influenced secretagogue-induced degranulation. Recombinant TNF-α was used, as described (19), for control ELAM-1 induction in explants.

Immunohistochemistry. For immunohistochemical staining, harvested explants were frozen in OCT compound (Miles Scientific) cooled in liquid nitrogen and stored at −80°C. Frozen specimens were cut in 4-μm cryostat sections, air-dried, fixed in acetone, and reacted for 0.5 hr at 37°C with the monoclonal antibody H4/18. Staining was accomplished by a three-step peroxidase-antiperoxidase technique (ref. 27) with 3,3′-diaminobenzidene as the chromogenic substrate. All incubation times were identical, and controls and blocking experiments were evaluated side-by-side with positive samples.

Morphological Studies. Routine transmission electron microscopy consisted of fixation of explants for 24 hr at 4°C. Specimens were washed in 0.1 M sodium cacodylate buffer, postfixed in 2% (wt/vol) osmium tetroxide for 1 hr, dehydrated in graded ethanol solutions to propylene oxide, and embedded in Epon. Examination of 1-μm sections (28) stained with toluidine blue and ultrathin sections stained with uranyl acetate and lead citrate was performed using light and transmission electron microscopy, respectively.

RESULTS

Mast Cell Degranulation in Skin Organ Cultures. The ultrastructure of human foreskin explants for the duration of culture (48 hr) was identical to that of noncultured human foreskin, with no evidence of medium or additive-related cytotoxicity. Mast cells in skin explants not exposed to secretagogue exhibited uniformly intact granules. These membrane-bound cytoplasmic granules were round, uniform in size and shape, and contained homogeneous electron-dense matrices (29) (Fig. 1*A*). All secretagogues tested produced mast cell degranulation in the skin explant system. MS resulted in widespread degranulation that affected all granules of all mast cells at 5 mmol/liter (Fig. 1 *B* and *C*); lower concentrations produced less prominent alterations in a dose-dependent manner. Degranulation was characterized by granule enlargement, membrane fusion of adjacent granules and between granule and plasma membranes, and electron lucency of granule matrices (Fig. 1*C*). These alterations were similar to those associated with IgE-mediated mast cell degranulation (30). Calcium ionophore and compound 48/80 resulted in similar granule alterations, although even at high concentrations (100 μg/ml and 10 mg/ml, respectively), ubiquitous degranulation was not observed. Exposure of explants beyond 45 min did not enhance degranulation for any concentration of secretagogue used. Accordingly, MS was chosen as the principal secretagogue for the subsequent experiments.

Anti-IgE at a maximum dilution of 0.78 mg/ml gave results similar to those obtained using calcium ionophore and compound 48/80. In keeping with previous studies (31), only foreskins incubated with IgE-containing medium (e.g., atopic or antigen-sensitized serum) resulted in anti-IgE-mediated degranulation. Importantly, control replicate explants incubated in rabbit IgG of similar protein concentration to the rabbit anti-human IgE used did not result in degranulation.

Mast cell granules were the only mast cell organelles to exhibit ultrastructural changes upon secretagogue exposure; adjacent mitochondria and endoplasmic reticulum were unaltered. Furthermore, fine structural alterations were not detected in epidermal cells, endothelial cells, fibroblasts, or macrophages, indicative that MS action was restricted to

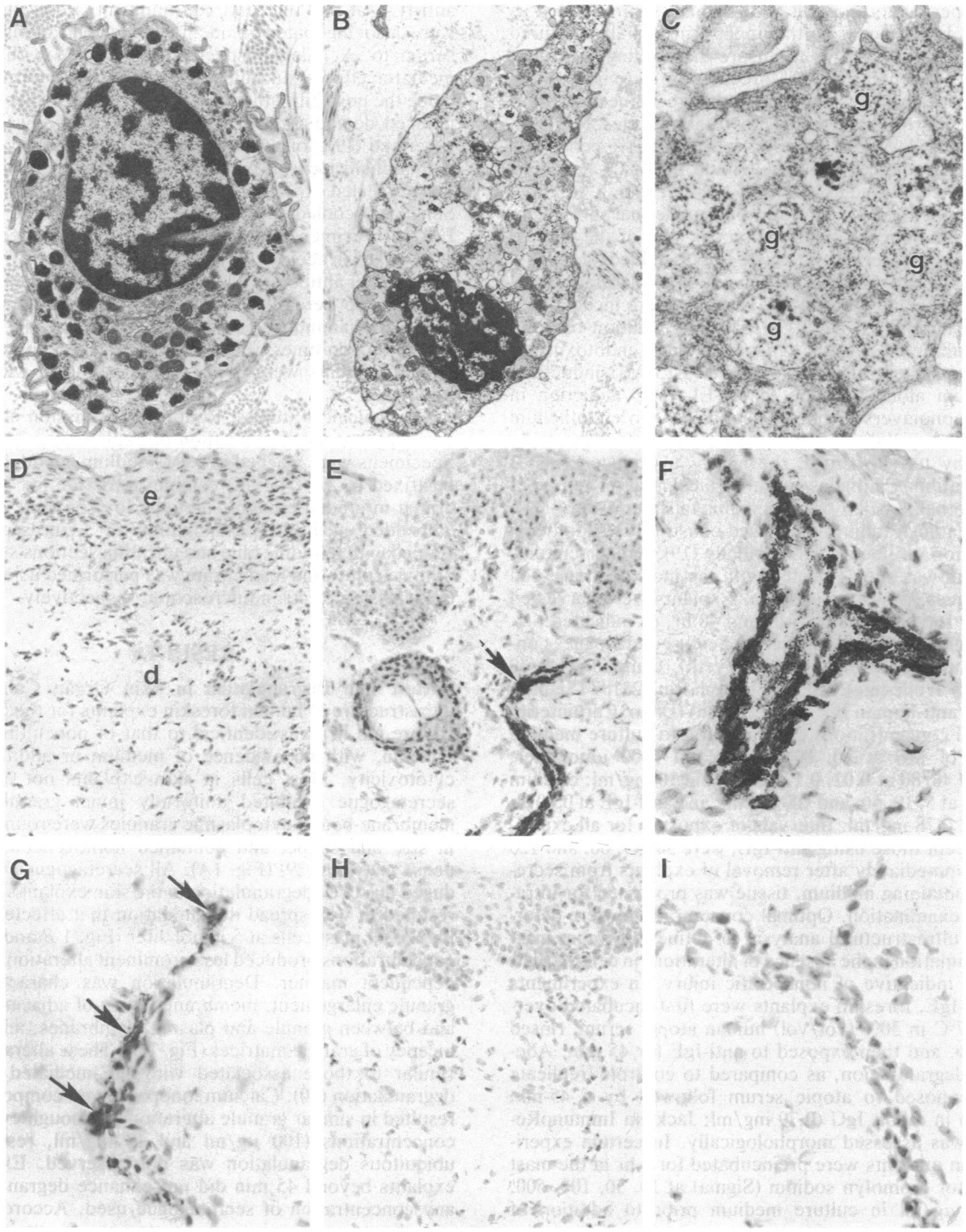

FIG. 1. Degranulation of dermal mast cells by MS and related induction of ELAM-1 in adjacent microvasculature. (*A*) Transmission electron micrograph of control dermal mast cell from newborn foreskin. These cells characteristically contain numerous (20–40) round electron-dense membrane-bound cytoplasmic granules of uniform size. (*B*) After MS exposure (45 min; 5 mM), mast cells were profoundly degranulated, as shown by granules that were enlarged, fused, and electron-lucent. (*C*) Individual granules (g) contained particulate amorphous material, characteristic of solubilization of granule contents. Similar, albeit less prominent, alterations were observed after exposure of skin explants to compound 48/80 and calcium ionophore. Granule alterations within mast cells were the only changes observed in explants at an ultrastructural level after exposure to secretagogues. This experimentally induced degranulation was qualitatively identical to that observed 4 hr after antigen challenge in cutaneous delayed hypersensitivity *in vivo*. (*D*) Control explant after 6 hr of culture (e, epidermal layer; d, dermis) exhibited no reactivity for H4/18 monoclonal antibody after immunohistochemical staining. (*E*) Explant cultured for 6 hr immediately after MS-induced mast cell degranulation. Prominent H4/18 reactivity, indicated by peroxidase-diaminobenzidine reaction product (arrow), is observed in a superficial dermal vessel. (*F*) Staining is characteristically circumferential after MS exposure. This reactivity indicates ELAM-1 induction in endothelium lining vessels surrounded by degranulated mast cells. Interrupted and generally less intense H4/18 reactivity (arrow) was typical of dermal vessels in explants cultured for 6 hr after compound 48/80 (*G*) and calcium ionophore exposure (data not shown). (*H*) Explant incubated with blocking antiserum to TNF-α, then exposed to MS, cultured for 6 hr, and stained immunohistochemically with H4/18. Abrogation of ELAM-1 induction (H4/18 reactivity) is apparent. (*I*) High magnification of dermal vessel from this experiment shows only hematoxylin counterstaining of endothelial nuclei. (For *A* and *B*, ×2800; for *C*, ×9600; for *D*, *E*, and *H*, ×160; for *F*, *G*, and *I*, ×400.)

mast cells. Externalization of mast cell secretory material into extracellular matrix that surrounds adjacent superficial dermal venules could be documented using this organ culture model (detailed report in preparation). This observation was interpreted as morphological evidence that secretagogue exposure resulted in delivery of mast cell mediators to surrounding structures.

Association of ELAM-1 Expression with Degranulation. H4/18 antibody reactivity was noted exclusively on endothelial cells lining venules in the superficial dermis in explants exposed for 45 min to MS and then cultured for 2 hr. This induction of ELAM-1 was not observed on any cell type in time-matched cultured explants or in foreskins prior to culture. ELAM-1 expression peaked at 6 hr (Fig. 1 *E* and *F*), declined by 24 hr, and was absent at 48 hr. These kinetics were identical to those characteristic of ELAM-1 induction by recombinant TNF-α in this organ culture model (19). Restriction of immunoreactivity to endothelial cells lining superficial venules has been confirmed by immunoelectron microscopy (19). Examination of these explants by electron microscopy indicated that ELAM-1 induction was consistently preceded by degranulation of mast cells normally situated about these superficial dermal venules (two to five mast cells per venule). Identical results were obtained using normal adult arm skin in which mast cells were degranulated with MS, indicating that these events were not peculiar to neonatal foreskin. In addition, similar data was also obtained when mast cells of foreskin were degranulated with compound 48/80 (10 mg/ml), calcium ionophore (100 μg/ml), or anti-IgE (0.78 mg/ml; Fig. 2), demonstrating that ELAM-1 induction was not a result of MS exposure (Fig. 1*G*). Calcium ionophore and compound 48/80 produced ELAM-1 staining that was generally less prominent than that associated with MS-induced degranulation. This observation is in keeping with our morphologic observations that compound 48/80 and calcium ionophore are less potent degranulating agents than MS in the organ culture system.

Abrogation of ELAM-1 Induction. Incubation of explants with polyclonal blocking antiserum to human TNF-α (500 units/ml), followed by MS-induced degranulation and organ culture (as described above), resulted in abrogation of ELAM-1 induction (Fig. 1 *H* and *I*). Similar strategies using antiserum to human IL-1 α and β (200 units/ml) failed to abrogate ELAM-1 induction. Morphologic studies confirmed mast cell degranulation in these experiments, precluding the possibility of interference by anti-TNF-α with the MS degranulation stimulus. Thus, release of endogenous TNF-α in association with mast cell degranulation appeared to be responsible for elicitation of endothelial activation, as defined by ELAM-1 induction. Abrogation of ELAM-1 induction also was accomplished by preincubation of explants with cromolyn sodium, a mast cell inhibitor (32, 33), indicating that endothelial activation was not a consequence of secretagogue exposure that was independent of degranulation.

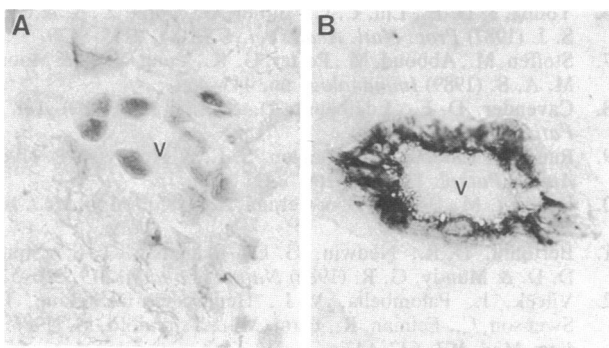

FIG. 2. Effect of IgE-mediated degranulation on induction of ELAM-1 in adjacent microvasculature. (*A*) Control dermal vessel (v) after preincubation in atopic serum and exposure to rabbit IgG. No H4/18 reactivity is seen in endothelial cells forming vascular lumen (v), and mast cells were not degranulated by morphologic criteria (data not shown). Endothelial nuclei are weakly counterstained with hematoxylin. (*B*) Endothelial cells lining dermal vessel (v) of explant preincubated with atopic serum and exposed to anti-human IgE (IgG fraction). Note strong endothelial H4/18 reactivity, indicating ELAM-1 induction. Morphologic evidence of mast cell degranulation was observed about these vessels. (×350.)

DISCUSSION

Mast cells are strategically positioned about superficial dermal venules (the site of most cutaneous inflammatory responses) for rapid and efficient delivery of preformed mediators as a result of degranulation. We demonstrate herein that degranulation of dermal mast cells is linked to the expression of an endothelial adhesion molecule for leukocytes (ELAM-1). Furthermore, the present study indicates that the immunoregulatory cytokine TNF-α may represent a critical endogenous mediator for ELAM-1 induction.

Several laboratories have provided evidence of TNF-α-like factors in mast cells. For example, cytotoxic capabilities of murine mast cells have been attributed to TNF-α (34, 35), and a cytotoxic factor with some immunological characteristics of TNF-α, but with differing molecular weight, has been identified in cloned murine mast cells (36). In human basophil/mast cells cultured from bone marrow progenitors, Steffen *et al.* (37) have documented TNF-α mRNA and immunoreactive TNF-α protein within cell cytoplasm and granules, respectively. It is unclear as to whether mast cell degranulation may also stimulate synthesis and release of TNF-α from another cell type (e.g., monocyte/macrophage) in the dermis. However, because the kinetics of ELAM-1 induction after mast cell degranulation are identical to those shown for direct addition of recombinant TNF-α to explant cultures (10), it is likely that mast cells release a factor with TNF-α-like properties upon degranulation.

Mast cell degranulation has been implicated in a variety of seemingly divergent pathological processes, including tumoricidal activity, fibroplasia and extracellular matrix remodeling, and host defenses against infectious agents. In addition to inducing ELAM-1, TNF-α has a number of pleiotropic effects, including enhanced endothelial cell metabolism, as measured by RNA and protein synthesis (38); establishment of chemotactic gradients for leukocytes (39); stimulation of collagenase and prostaglandin E_2 production (40), stimulation of osteoclasts and bone resorption (41), induction of fibroblast proliferation (42), and enhancement of eosinophil toxicity to *Schistosoma mansoni* larva (43). Thus, acute and chronic release of TNF-α-like molecules in association with degranulation may aid in explaining the role of mast cells in a wide range of tissue responses.

Another implication of these findings is that exogenous (e.g., morphine) and potentially endogenous (e.g., anti-IgE) factors recognized to affect mast cell degranulation may elicit pathways that stimulate endothelial–leukocyte interactions fundamental to clinical expression of certain inflammatory dermatoses. It is also of interest that endogenous neurogenic signals that trigger degranulation, such as substance P, have been implicated in the induction of experimental cutaneous inflammation (44). Considered within the context of the present findings, a potential link is established between neural factors, mast cell-associated proinflammatory mediators (TNF-α or similar molecules), and the development of a cellular inflammatory response. Such a link could have clinical significance in the commonly observed exacerbation of many dermatoses, such as psoriasis and atopic disease, by emotional stress.

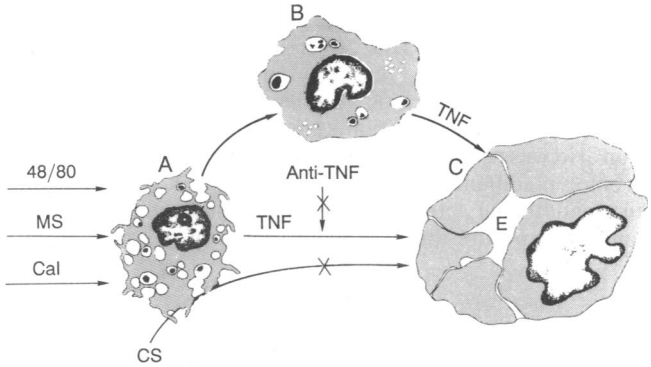

FIG. 3. Summary of effects of mast cell degranulation in human foreskin organ cultures. Degranulation of dermal mast cell (*A*) surrounding superficial dermal venule (*C*) by secretagogues, compound 48/80, MS, or calcium ionophore (arrows to left of *A*), may result in direct or indirect (by monocyte/macrophage) (*B*) release of TNF-α. This, in turn, results in rapid and transient expression of ELAM-1 on the endothelial surface. These events are experimentally abrogated by the mast cell inhibitor cromolyn sodium and by blocking antiserum to TNF-α. E, ELAM-1; CaI, calcium ionophore; CS, cromolyn sodium.

In summary, this study indicates that mast cell degranulation is linked by cytokine-dependent pathways to induction of ELAM-1 in dermal microvessels (Fig. 3). These findings help explain the central role of mast cell secretion in the initiation of cutaneous inflammation. As gatekeepers of the microvasculature, mast cells should be critical targets of therapeutic strategies that seek to prevent or ameliorate the earliest phases of the cellular inflammatory cascade.

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