ORIGINAL ARTICLE

Augmented Inflammatory Responses and Altered Wound Healing in Cathepsin G–Deficient Mice

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Background: Cathepsin G is a neutral serine proteinase that exists primarily in azurophilic granules of neutrophils, but also as a proteolytically active membrane-bound form. While the specificity and many in vitro biological activities have been described for cathepsin G, little is known about the role of this enzyme in neutrophil function in vivo, particularly as it applies to the wound-healing process.

Objective: To determine the role of cathepsin G in cutaneous tissue repair by examination of full-thickness incisional wound healing in mice with a null mutation for cathepsin G.

Methods: Paired, full-thickness linear incisions were made on the backs of cathepsin G +/+ and cathepsin G –/– mice, and wound tissue was harvested at days 1, 2, 3, 5, 7, 10, and 14 after wounding. Neutrophil influx, myeloperoxidase activity, and migration were examined using light microscopy, the myeloperoxidase assay, and modified Boyden chamber technique, respectively. Wound-breaking strength was measured using tensiometry.

Results: The absence of cathepsin G led to a 42% decrease in wound-breaking strength at day 7 after wounding (n = 28; $P<.002$), which returned to the level of control mice by day 10 after wounding. Wound tissue sections in mice lacking cathepsin G also showed a 26% increase in neutrophil myeloperoxidase activity (n = 12; $P = .001$) and an 18% increase in neutrophil influx (n = 14; $P = .002$) at day 3 after wounding. Wound fluid collected on day 5 after wounding from cathepsin G–deficient mice attracted 58% more neutrophils than wound fluid collected from control mice (n = 4; $P<.05$).

Conclusions: Neutrophil cathepsin G is important during the early inflammatory stage of wound healing. Cathepsin G may be involved in processing 1 (or more) soluble mediator(s) in the wound milieu that is responsible for neutrophil chemotaxis. Our findings suggest that tight regulation of inflammation is necessary to prevent impaired healing during early tissue repair.

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THE WOUND-healing process is best described as a dynamic interplay between an orderly procession of cell types, extracellular matrix, formed blood elements, and soluble mediators. Traditionally, wound healing has been divided into 3 sequential stages: inflammation, cellular proliferation with granulation tissue formation, and tissue remodeling and maturation. The inflammatory stage has long been recognized as integral to creating a suitable environment for the succeeding cellular events involved in tissue reparation. During early inflammation, neutrophils actively destroy contaminating microorganisms and provisional matrix by using degradative enzymes and generating oxygen radicals during phagocytosis.[1,2] However, in the absence of a significant inflammatory stimulus, neutrophils are traditionally regarded as playing an insignificant role in tissue repair,[3] with the macrophage being held as the primary cellular effector of healing.[4] The persistence of microorganisms or indigestible foreign matter can, though, lead to a protracted neutrophilic response, delaying (or even impairing) healing.[5,6] The secretion of distinct proteins contained within neutrophilic granules is presumed to be important in mediating this altered healing.

Cathepsin G is a neutral serine proteinase packaged in the azurophilic granules of mature neutrophils.[7] On activation, neutrophils secrete cathepsin G, as well as maintaining some proteolytically active enzyme bound to their outer membrane.[8] Among the activities of cathepsin G are platelet activation,[9] microbicidal activity,[10] conversion of angiotensin I to angiotensin II,[11] and cleavage of a variety of clotting factors.[12] However, its ability to modulate proinflammatory cytokines and degrade extracellular matrix components makes it superficially appealing for wound-healing studies. It has been shown to process the neutrophil chemoattractants tumor necrosis factor α (TNF-α),[13] interleu-

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MATERIALS AND METHODS

MICE

Cathepsin G –/– (test) and cathepsin G +/+ (control) mice were generated using selective gene targeting (D.M.M., S. Shapiro, T.J.L. unpublished data, 1998). The embryonic stem cell line used was RW-4, derived from 129/SVJ mice. These cells were injected into C57/BL6 mice, and the chimeric agouti offspring were bred to BL6 mice. The animals from this breeding were analyzed for the cathepsin G mutation, and heterozygous pairs were bred to produce the homozygous (null) mutant mice. All cathepsin G mice used in this experiment were therefore based on a mixed 129/SVJ × C57/BL6 background. In all experiments, both test and control groups were composed of age- and sex-matched littermates. All experimental procedures were approved by the Animal Care and Use committees of Northwestern University, Chicago, Ill, and Washington University, St Louis, Mo.

WOUNDING

Cathepsin G –/– and +/+ mice, weighing 23 to 28 g, were acclimated and housed under standard conditions prior to wounding. Mice were anesthetized using 0.05 mg/g of ketamine hydrochloride solution intraperitoneally and 0.10 mg/g of xylazine hydrochloride solution intraperitoneally, shaved with electric clippers, and cleansed with a 70% alcohol solution. Using a sterile technique, paired 3.0-cm full-thickness linear incisions were made on their backs at a distance of 1.5 cm apart. Incisions were closed with a running polypropylene suture. After the operation, animals were housed in individual cages under standard conditions and checked daily for any signs of duress. On tissue harvest, animals were killed using lethal intracardiac injection of pentobarbital.

BIOMECHANICAL TESTING

On days 7, 10, and 14 after the wounds were made, 28 wound strips were harvested from both cathepsin G –/– (test) and cathepsin G +/+ (control) groups. Wounds were cut in a standardized fashion using a template, creating strips 0.5 cm long and 1.25 cm wide. The peak load of each wound strip at break was measured using a tensiometer (Instron, Canton, Mass) and previously established methods.[20] Additionally, standardized tissue strips from unwounded cathepsin G –/– and cathepsin G +/+ mice were tested to determine whether there were any inherent differences in native tissue.

HISTOMORPHOMETRY/LIGHT MICROSCOPY

On days 1, 2, 3, 5, 7, 10, and 14 after wounding, 14 wound strips from both cathepsin G –/– and cathepsin G +/+ mice were fixed in 4% paraformaldehyde solution (Columbia Diagnostics, Springfield, Va), embedded in paraffin (Surgipath, Richmond, Ill), and cut into 7-µm sections. Each section was stained with May-Grünwald and Giemsa stains (Sigma Chemical Co, St Louis, Mo) and viewed with a light microscope (Nikon, Tokyo, Japan) at ×400 magnification. Based on cell morphologic features and without knowledge of genotypes, 2 independent observations of neutrophil densities in the 2 most cellular high-power fields (HPFs) per each wound tissue section were made. Additionally, wound tissue sections were examined for quantitative differences in mononuclear cells.

MYELOPEROXIDASE ASSAY

On days 3, 5, and 7 after wounding, 12 wound sections from cathepsin G –/– and cathepsin G +/+ mice, each weighing approximately 50 mg, were washed in phosphate-buffered saline solution and homogenized in 500 µL of 0.1-mol/L Tris chloride–buffered solution with a pH of 7.6, 0.15-mol/L sodium chloride solution, and a 0.5% hexadecyltrimethylammonium bromide solution (Sigma Chemical Co) using a tissue grinder (Wheaton, Millville, NJ). Homogenates were then sonicated for 15 seconds, freeze-thawed 3 times, and centrifuged at 10 000 rpm at 4°C. Supernatant fluids were collected, normalized for total protein content (Biorad assay), and assayed for myeloperoxidase activity using a modification of the method described by Trush et al[21] by adding 10 µL of supernatant fluid to 200 µL of 50-mol/L potassium phosphate–buffered solution with a pH of 6.0, containing 0.2% of *O*-dianisidine dihydrochloride (Sigma Chemical Co) and 0.0001% hydrogen peroxide solution, measuring the absorbance of 460 nm at 4 minutes.

NEUTROPHIL MIGRATION

Using a sterile technique, four 0.5-cm^3 polyvinyl alcohol sponges (Mpact, Eudora, Kan) were placed in subcutaneous pockets beneath the skin on the back of cathepsin G –/– and cathepsin G +/+ mice. At days 1, 2, 3, and 5 after wounding, sponges were recovered from the animals and centrifuged at 5000 rpm at 4° for 10 minutes. Wound fluid was then taken as the supernatant fluid from the centrifugate, diluted 1:10, and normalized for total protein content (Biorad assay). Chemotactic activity was measured using a Boyden chamber[22]: 30 µL of wound fluid was separated from 50 µL of normal mouse neutrophils at a concentration of 3×10^6 neutrophils/mL using a 2.0-µm polycarbonate membrane (Poretics Corporation, Livermore, Calif). After incubation at 37°C in 5% carbon dioxide for 30 minutes, the membrane was removed, stained, washed, and fixed on a slide. Neutrophils able to move through the membrane pores were identified and counted at ×400 original magnification.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD. Cathepsin G –/– and cathepsin G +/+ groups were compared using paired Student *t* tests. Significance is claimed for a 2-tailed $P<.05$.

kin 1,[14] and interleukin 8[15] and to directly cleave collagen,[16] cartilage proteoglycans,[17] elastin,[18] and fibronectins.[19]

Mice generated with a null mutation for cathepsin G display healthy growth and development, fertility, and hematopoiesis (D.M.M., S. Shapiro, PhD, T.J.L. unpublished data, 1998). Neutrophils derived from these mice have healthy morphologic characteristics, azurophilic granule composition, and phagocytic and superoxide-producing capacities (D.M.M., S. Shapiro, PhD, T.J.L., unpublished data, 1998).

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Given the known biochemical properties of cathepsin G, particularly its ability to degrade extracellular matrix but also its capacity to degrade proinflammatory cytokines, we chose to investigate the wound healing of full-thickness cutaneous wounds created on the backs of mice with a null mutation for cathepsin G. Our specific goals were (1) to determine if the absence of cathepsin G changed the progression of the inflammatory stage of wound healing, particularly with regard to neutrophil activity, and (2) to determine whether such a change would effect an alteration in subsequent wound healing.

RESULTS

BREAKING STRENGTH OF WOUND STRIPS

To determine whether there were any inherent differences in native tissue from cathepsin G –/– and cathepsin G +/+ mice, the peak load at break of standardized unwounded tissue strips was measured and found to show no difference (n = 14; $P>.05$; **Figure 1**). We proceeded to test the peak load at break of standardized wound strips harvested from cathepsin G –/– and cathepsin G +/+ mice on days 7, 10, and 14 after wounding (Figure 1). On day 7 after wounding, the breaking strength of wound tissue strips from cathepsin G –/– mice was 89.7 ± 33.1 g, while that of wound tissue strips from cathepsin G +/+ mice was 153.7 ± 50.1 g, a difference of 42% (n = 28; $P<.002$). At day 10 after wounding, when the healing wounds were well into the proliferative stage, the difference between test and control groups failed to persist (n = 28; $P>.05$). At day 14 after wounding, as the healing wounds continued to mature, a difference between the test and control groups was also not demonstrated (n = 28; $P>.05$).

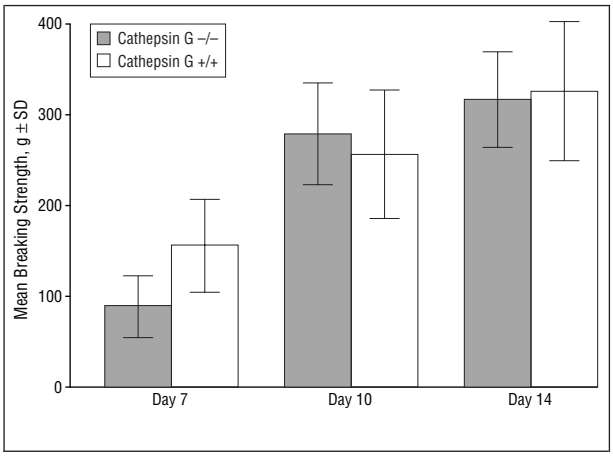

Figure 1. *Peak load at specimen break of standardized wound strips. On day 7 after wounding, cathepsin G –/– demonstrated a 42% decrease in breaking strength vs controls (n = 28; P<.002). The impairment in healing failed to persist for days 10 and 14 after wounding. There were no differences in peak load at specimen break of unwounded tissue strips between groups (data not shown).*

DENSITY OF NEUTROPHILS IN TISSUE SECTIONS

On days 1, 2, 3, 5, 7, 10, and 14 after wounding, using an HPF and without knowledge of genotype, neutrophil counts were assessed in hematoxylin-eosin–stained wound tissue sections from cathepsin G –/– and cathepsin G +/+ mice by 2 observers (**Table 1**). On days 1 and 2 after wounding, neutrophils were the predominant wound cell in both the test and control groups, but failed to show any quantitative differences (n = 14; $P>.05$). On day 3 after wounding, neutrophils were still the predominant wound cell in both the test and control groups; however, the cathepsin G –/– wound tissue sections demonstrated 18% more neutrophils with an HPF than did that of cathepsin G +/+ wound tissue sections (n = 14; $P = .002$; **Figure 2**). Beyond day 3 after wounding, neutrophils became less conspicuous in both test and control wound tissue sections, essentially becoming absent by day 7 after wounding. No statistically significant differences were demonstrated in the amount of neutrophils using an HPF between genotypes beyond day 3 ($P = .03$). Also, no differences were noted in overall wound cellularity between genotypes beyond day 3, and no differences were noted between quantities of mononuclear cells between genotypes at any time. There was no evidence of infection in any wound tissue sections examined.

NEUTROPHIL MYELOPEROXIDASE ACTIVITY

The myeloperoxidase activity of wound tissue was assayed on days 3, 5, and 7 after wounding to correlate neutrophil counts using an HPF with a semi-quantitative measure of neutrophil abundance (**Table 2**).Wound tissue obtained from cathepsin G –/– mice at day 5 after wounding demonstrated a 26% increase in myeloperoxidase activity compared with cathepsin G +/+ mice (0.346 ± 0.041 absorbance units vs 0.275 ± 0.055 absorbance units, both groups; n = 8; $P = .0002$). On day 5 after wounding, neutrophil myeloperoxidase activity of wound tissue from both test and control groups failed to show a difference (n = 8;

Table 1. Neutrophil Density in Healing Wounds*

	After Wounding, d				
Genotype	1	2	3†	5	7
Cathepsin G–/–	122.0 ± 15.9	178.9 ± 15.3	219.4 ± 23	43.4 ± 13.8	<10
Cathepsin G+/+	113.2 ± 13.1	171.9 ± 15.8	185.8 ± 26.3	39.2 ± 16.2	<10

**Neutrophils were counted in embedded paraffin sections of wound tissue stained with May-Grünwald and Giemsa stains. Numbers reflect the mean ± SD density of neutrophils based on cell morphologic characteristics of 2 independent observations at ×400 original magnification in the 2 most cellular high-power fields per wound tissue section, without knowledge of genotype. Fourteen cathepsin G –/– mice and 14 cathepsin G +/+ mice were analyzed.*

†P<.002

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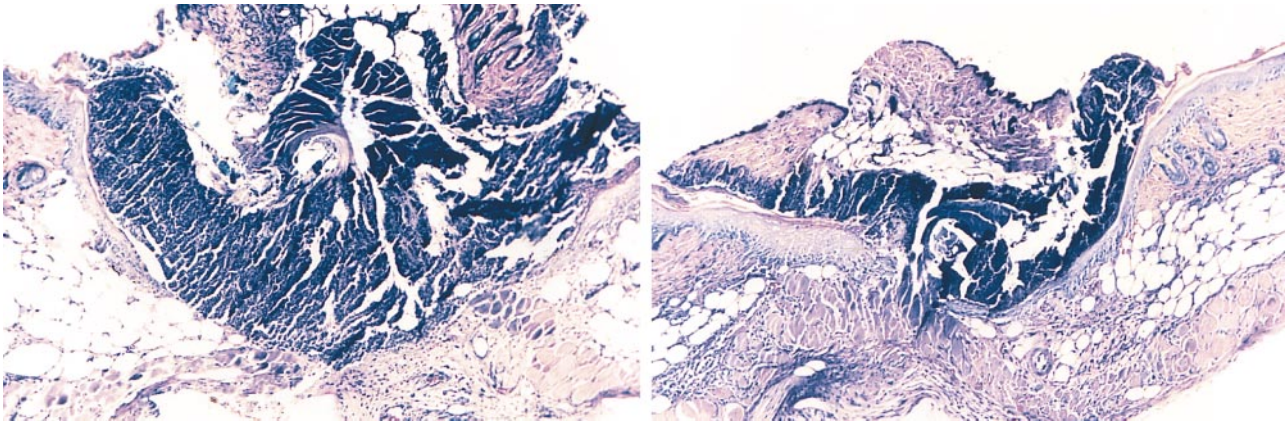

Figure 2. *Histological sections of cathepsin G –/– and cathepsin G +/+ wound areas. In May-Grünwald– and Giemsa–stained sections on day 3 after wounding, cathepsin G–deficient mice (left) demonstrated a greater density of neutrophils using a high-power field than did control mice (right). This difference failed to persist. In both wounds, effete acute inflammatory cells and cellular debris were found in the desiccated upper portions of the scab, while active inflammatory cells were present just beneath these portions. The scab was in the process of being dissected from the healing wounds as reepithelialization occurred. S indicates scab; E, epidermis; and D, dermis (original magnification ×100).*

$P>.05$). By day 7 after wounding, myeloperoxidase activity was virtually absent from both test and control groups and again failed to show a difference (n = 8; $P>.05$).

CHEMOATTRACTANT ACTIVITY IN WOUND FLUID

The chemotactic responses of normal neutrophils to wound fluid obtained from cathepsin G –/– and cathepsin G +/+ mice were evaluated using a Boyden chamber. There were no notable differences in volumes of wound fluid and amounts of protein obtained from each of the groups. After diluting 1:10 and correcting for protein content, wound fluid obtained on day 3 after wounding from mice lacking cathepsin G attracted 58% more neutrophils than did wound fluid harvested from control mice at the same time (187 ± 37 vs 118 ± 49 neutrophils per HPF, respectively; n = 4 samples of wound fluid; $P<.05$). The chemotactic responses of neutrophils to wound fluid collected from both genotypes at days 1, 2, and 5 after wounding were not different ($P>.50$)(n = 4, for all groups).

Table 2. Myeloperoxidase Activity in Healing Wounds*

	After Wounding, d		
Genotype	3†	5	7
Cathepsin G–/–	0.346 ± 0.041	0.122 ± 0.060	0.101 ± 0.037
Cathepsin G+/+	0.27 ± 0.055	0.110 ± 0.056	0.108 ± 0.028

**Neutrophil myeloperoxidase activity measured as change in absorbance units at 4 minutes (wavelength, 460 nm). Twelve cathepsin G –/– mice and 12 cathepsin G +/+ mice were analyzed.*
†$P<.002$

COMMENT

While the macrophage is widely held as being the primary cellular effector of tissue reparation,[4] the neutrophilic leukocyte (and its granular contents) has been traditionally regarded as playing a nonessential role in uncomplicated wound healing. Studies using antineutrophil serum to induce neutropenia in guinea pigs led to no change in wound cellularity and granulation tissue formation when examined on days 7 through 10.[3] However, more recent studies have examined the role of the neutrophil in early wound repair. The use of antineutrophil serum in rats led to the prevention of the usual decrease in intestinal anastomotic suture-holding capacity during the early inflammatory stage of wound healing.[23] The use of broad-spectrum serine proteinase inhibitors and oxygen-derived free radical scavengers also prevented the usual decrease in anastomotic suture-holding capacity (although not as much as with antineutrophil serum).[24] From these studies, it is clear that the neutrophil does play an important role in tissue repair during the inflammatory stage of wound healing, especially as it pertains to the dehiscence of surgical wounds.

By induction of a null mutation for cathepsin G in mice, we were able to look more specifically at the role of this particular serine proteinase inhibitor in the process of cutaneous wound healing. On day 7 after wounding, a substantial decrease in wound-breaking strength was demonstrated in test mice; this defect was corrected at days 10 and 14 after wounding. An examination of wound tissue sections at day 3 after wounding showed an increase in neutrophil density and neutrophil mass (as measured by myeloperoxidase activity) in test mice. We postulate that the wound impairment is, in part, a consequence of excessive neutrophil infiltration and protracted inflammation that subsequently leads to a decrease in wound-breaking strength. The mechanism could include excessive proteolysis and/or oxygen free radical damage caused by neutrophilic enzymes other than cathepsin G. We expect that wound impairment would also have been detected earlier, but breaking strength is not reliably assessed at earlier points, since the fibroplastic stage of wound healing is not sufficiently under way.[25] Additionally, wound fluid collected from test mice on day 5 after wounding caused increased neutrophil chemotaxis in migration assays. This lag time indicates that there may be something unique about the cathepsin G –/– wound environment during inflammation that leads to increased neutrophil chemotaxis and explains the significantly increased density of neutrophils seen in cathepsin G–deficient wounds. It is important to em-

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phasize that there is a time lag in the collection of wound fluid from polyvinyl sponges. That is, wound fluid collected on day 5 after wounding is in fact a collection of wound fluid elaborated during the early inflammatory process. This result would account for the increase in neutrophil density and myeloperoxidase activity seen in wound tissue sections on day 3 after wounding but not beyond.

Cathepsin G may play a role in degrading proinflammatory cytokines that are elaborated in the wound fluid during inflammation and these cytokines serve as neutrophil chemoattractants. In the absence of cathepsin G, a protracted chemotactic signal may persist, leading to an increased influx of neutrophils and subsequent neutrophil-mediated tissue damage. Studies have shown that cathepsin G can play a role in the degradation of TNF-α and interleukin 8.[11-13,18] If such cytokines were present in excess in the wound milieu, they could account for the protracted signaling, as well as the activation of other inflammatory pathways leading to tissue damage. For example, TNF-α has been found to decrease collagen[26] and increase collagenase production[27] in vivo, and both interleukin 1β and TNF-α are potent inducers of matrix metalloproteinase expression.[28] Additionally, the association of impaired healing with increased proinflammatory cytokines, particularly TNF-α,[29] already exists in models of sepsis. So clearly, a portion of the wound impairment observed in cathepsin G–deficient mice may be from downstream effects of such agents and not just the direct effects of increased neutrophil activity. Monocytes and macrophages are the primary source of these cytokines; although these are not the predominant cell types during the early inflammatory stage, they are still present in sizable quantities soon after wounding.[30] Because macrophages do become the predominant cell type in the wound (typically by day 5 after wounding), they effectively phagocytose the remaining apoptotic neutrophils.[31] In the cathepsin G–deficient mice, our data suggest that the exuberant neutrophil influx ceases when macrophages remove the neutrophils from the wound. Consequently, the cathepsin G test wounds ultimately return to the level of wound healing attained in control wounds.

Collectively, these data suggest that, just as wound healing is impaired by diminishing the inflammatory response (eg, via corticosteroid therapy, chemotherapeutics, and radiation treatment), it is also impaired by an excessive inflammatory response. Our findings underscore the importance of a stringently regulated inflammatory response in tissue repair.

In future experiments, we will attempt to define the factor(s) in the wound fluid that leads to exuberant neutrophil influx and inflammation, as well as examine how this affects the proteinase profile in the healing wound. Also, we intend to test the hypothesis that cathepsin G is integral to early wound healing by demonstrating that the addition of exogenous cathepsin G will revert the impaired phenotype to that of the wild type.

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