The Wound Is a Possible Source of Posttraumatic Immunosuppression

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MATERIALS AND METHODS
Generation of Wound Fluid

Male Lewis rats (Harlan Sprague-Dawley, Indianapolis, Ind), weighing 225 to 275 g, underwent a 7-cm dorsal skin incision with subcutaneous placement of sterile, moist polyvinyl alcohol sponges (Unipoint Industries, High Point, NC). The incisions were closed with surgical stainless-steel staples. The rats were killed 10 days after wounding, and the sponges were removed and cleared of the surrounding granulation tissue. The sponges were squeezed with sterile forceps, and the fluid was collected into conical tubes. The fluid was rendered acellular by centrifugation at 150 g for 10 minutes. The cell-free supernatant was passed through a 0.22-µm filter, aliquoted, and stored frozen at -70°C (henceforth called wound fluid). For controls, rat blood was obtained by cardiac puncture and the serum was also stored frozen at -70°C.

Cecal Ligation and Puncture

Forty-six male Sprague-Dawley rats weighing between 275 and 225 g were individually caged and allowed 1 week of acclimatization to our laboratory conditions. The rats were given atropine (0.04 mg/kg of body weight subcutaneously) and droperidol and fentanyl citrate (Innovar Vet; 0.1 mL/kg of body weight intramuscularly) and underwent cecal ligation and puncture with a single 23-gauge needle according to the technique of Wichterman et al.6 This puncture size was noted in preliminary experiments to result in approximately 50% mortality at 4 days. The abdominal musculature was closed with a running 8-0 silk suture, and the skin was approximated with surgical staples. All animals were resuscitated with 5 mL of normal saline given subcutaneously. Immediately after cecal ligation and puncture, rats were randomized to receive intravenously either 1 mL of wound fluid (adjusted to 10 mg of protein per milliliter) or 1 mL of rat serum (adjusted to similar protein content). Injections were given intravenously every 12 hours for 5 days.

Thymic Lymphocyte Mitogenic Assay

Wound fluid immunosuppressive activity in vitro was tested in a thymic lymphocyte mitogen assay. Briefly, thymus glands were removed from uninjured male Lewis rats, and single thymic lymphocyte suspensions were obtained by passing the thymus glands sequentially through 80 and 100 mesh stainless-steel screens. The lymphocyte suspension was washed twice with medium and brought to a concentration of 2.5 x 10⁶ cells per milliliter of RPMI 1640 medium supplemented with 2.5 mM/L glutamine, 50 U/mL of penicillin, 50 mg/L of streptomycin, and 10% heat-inactivated fetal calf serum. Cell viability as tested by trypan blue exclusion exceeded 95% in all cases. Triplicate cultures of 5 x 10⁵ thymic lymphocytes were set up in microtiter plates and mitogenesis in response to phytohemagglutinin (80 µg per well) was assayed (this concentration has been found to give optimal proliferative responses under our laboratory condi-
The wound fluid was tested for the presence of three cytokines as follows: (1) Tumor necrosis factor α was assayed with the use of the L929 cell line, which detects bioactivity at a lower limit of 500 ng/L. (2) γ-Interferon was tested with the use of a vesicular stomatitis virus cytopathic assay in the murine L929 cell line. This assay recognizes both murine and rat γ-interferon at a minimal concentration of 4 U/mL. (3) Transforming growth factor β was tested by using a rabbit polyclonal antibody which, at a concentration of 10 μg, neutralizes the growth-promoting effect of 1 ng/mL of transforming growth factor β on NRK49F cells (R&D Systems, Minneapolis, Minn.). In the thymic lymphocyte mitogenesis assay, cultures were incubated with 10% vol/vol wound fluid or wound fluid plus the anti-transforming growth factor β antibody at a concentration of 2.5 μg per well.

**Data Analysis**

Data were entered into a computer (Macintosh II, Apple Computer Inc, Cupertino, Calif) and analyzed by means of the StatViewII program (Abacus Concepts, Berkeley, Calif). All data are reported as mean ± SEM. Comparison of survival rates was accomplished by means of the χ² method. All other statistical analyses used Student’s t test.

**RESULTS**

Wound fluid was obtained from four groups of 10 to 12 Lewis rats. The wound fluids inhibited normal thymic lymphocyte mitogenesis in response to phytohemagglutinin (controls, 43 959 ± 5755 cpm; wound fluid–treated rats, 3387 ± 928 cpm). No evidence of cytotoxicity was noted with the use of trypan blue exclusion.

Intravenous administration of wound fluid every 12 hours after cecal ligation and puncture markedly increased the mortality of the animals (Figure). Starting 72 hours after the procedure, the mortality of the wound fluid–treated animals was statistically higher than that of the animals treated with normal rat serum. Examination of the dead animals and of the animals surviving after 5 days revealed murky peritoneal fluid with walled-off abscesses. Cultures showed the presence of the usual colonic polymicrobial flora. No gross differences were noted between control and wound fluid–treated animals.

To understand the mechanism by which wound fluid induces host immunosuppression, we examined wound fluid obtained from 10-day-old wounds for the presence of three cytokines that have been implicated in both wound healing and host immunosuppression. With the use of highly specific bioassays, no tumor necrosis factor α or γ-interferon bioactivity could be detected. In addition, polyclonal rabbit anti-transforming growth factor β antiserum did not abrogate the immune inhibitory activity of wound fluid on thymic lymphocyte blastogenesis (control, 41 150 ± 4778 cpm; 10% wound fluid–treated rats, 3047 ± 444 cpm; controls with anti–transforming growth factor β, 48 687 ± 4551 cpm; and controls with 10% wound fluid and anti–transforming growth factor β, 1515 ± 274 cpm).

**COMMENT**

The above experiments demonstrate that fluid obtained from 10-day-old healing wounds greatly impairs host resistance to an acute septic insult resulting in significantly higher mortality. This suggests that during the course of normal wound repair, factors are generated that can induce systemic immunosuppression. In the present study, the wound fluid, together with the immune inhibitory factors that it contains, was administered systemically and thus exerted its deleterious effect on the treated rats. There is no direct evidence that such a systemic spillover occurs in vivo. We have postulated that this indeed may occur after extensive tissue injury and are planning experiments to examine the occurrence of such a phenomenon. Systemically administered wound fluid does not seem to be toxic to healthy animals even when administered for 21 days intraperitoneally.

We previously showed that wound fluid markedly impairs in vitro lymphocyte proliferation in response to lectins or allogeneic stimulation. The effect of the wound fluid inhibitory factor seems to be one of selectively impairing interleukin (IL) 2-dependent immune responses. The 10-day wound fluid is not cytotoxic, and it even enhances fibroblast proliferation. The immune inhibition of the wound fluid can be duplicated by highly purified wound mononuclear cells by themselves or by their conditioned medium. Such local immune inhibition probably serves a dual function. First, it may help in preventing the host from developing autoreactive T-cell clones to a variety of proteins that are released or generated during injury and healing. Second, it may act as a down-regulating mechanism to the exuberant cellular activation that is necessary for initiating successful wound repair. There is in vivo evidence that wounds are sites that immunologically react differently from other body sites. One elegant study demonstrated in guinea pigs that small inocula of methylcholanthrene-induced liposarcoma cells, which do not give rise to tumors when inoculated into normal skin, lead to tumor formation and death when inoculated into 3-, 9-, and 11-week-old wounds.

The nature of the wound immune inhibitory factor is not fully known. We previously determined that it is a protein with an approximate molecular weight of 100 kD, heat stable (60°C, 1 hour), and neuraminidase sensitive, which suggests a high degree of glycosylation. In the present experiments we examined wound fluid for the presence of cytokines, which have been implicated in wound healing and which also have direct effects on the immune system. Tumor necrosis factor α, a product of activated macrophages, was not found in the wound fluid. γ-Interferon and transforming growth factor β are both products of activated lymphocytes that have been...
shown to participate in wound healing and to possess immune inhibitory activity. However, we could detect no evidence of their presence in the wound fluid. Wound fluid, as mentioned above, has been shown to inhibit IL-2–dependent immune reactions. Interleukin 2 has been shown to have a beneficial effect on the response of animals to an acute septic challenge, and perhaps this anti-IL-2 activity of wound fluid may partly explain its deleterious effect in the present experiments.13,14

The wound fluid immune inhibition appears 7 to 10 days after wounding and is not present at earlier times.1 This has also been shown in humans that immunosuppression, whether measured by the appearance of serum suppressive factors or of T-suppressor lymphocytes, occurs 5 to 14 days after injury.1 Clinically this also corresponds to the period when severely injured patients are at their maximal risk for sepsis and sepsis-related multiple organ system failure.15

In conclusion, during the normal process of wound healing there is generation of local immunosuppressive factors that, when administered systemically, can alter host responses to infection. Thus, the wound may, under circumstances of extensive injury, generate factors that can alter systemic host immune responses.

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References


Discussion

RICHARD SIMMONS, MD, Pittsburgh, Pa: Unlike those of you who are not self-centered, I have always liked papers that tend to support favorite hypotheses that I have held without data for a long time. And I have always hypothesized that the products of a healing wound were designed to stay there, and if they are released by, for example, oversea&reg;es rese&deg;agation then they might turn the whole body into a healing wound yielding the surgical intensive care unit or sepsis patient; therefore, I like this one.

The authors postulate that the wound contains factors that play a role in the regulation of wound healing and that are detrimental to overall host defense. They certainly show that very clearly, that animals that receive 10-day wound fluid die more often with experimental peritonitis. This correlates with an inhibitory effect on in vitro lymphoid tests. Neither these data nor other data in this field prove that the inhibition of lymphocyte function influences the response to intraperitoneal infection. The data on IL-2 protection against intraperitoneal infection, for example, work only if you give the IL-2 intraperitoneally. Systemic IL-2 does not work.

I have a number of technical questions:

We do not know what concentration of wound fluid was used in the in vivo assays, perhaps the biocomposite that used in the in vivo administration. Almost all the lymphoid tests that we do in vitro are done in very low concentrations of mouse serum, and if you simply raise the concentration of mouse serum you get inhibition. In fact, mouse serum is very immunosuppressive at concentrations greater than 2%. I presume that is true of rat serum. Dr Ford just showed that wound fluid contains a potent inhibitor of IL-2, and so does serum, and there is a poster that beautifully shows that IL-2 receptor is present in high quantities in the serum of traumatized patients. I think it possible that you have an IL-2 receptor overabundance in this wound fluid that acts as the immunosuppressant in vivo.

With respect to the finding that the injection of wound fluid results in higher mortality than rat serum, I have also a few questions. How did you choose the dose of protein, 10 mg of protein per milliliter? Did higher or lower doses change your impression?

Did you study the wound fluid for endotoxin before you gave it?

Did you inject the serum of sponge-bearers rats also? In other words, did you find any evidence that there was in fact a spillover of this immunosuppressant factor?

The possible effect of the plastic material in the sponge has been raised by a previous discussant. You used a polyvinyl alcohol sponge. Polyvinyl chloride is a plastic that continues to stimulate an acute response that does not ever subside. Could polyvinyl alcohol have a similar effect? If so, the wound fluid might differ from that which surrounds a less reactive biomaterial.

CAROL MILLER-GRAZIANO, MD, Worcester, Mass: I am a little confused about the assay you were using. It seemed to me that you were using a splenocyte proliferation assay; in other words, you were using, spenocytes and phytohemagglutinin. It is my understanding of the assay you were using. So maybe I just misunderstood what you said there.

I have a number of technical questions:

Did you study the wound fluid for endotoxin before you gave it?

How did you choose the dose of protein, 10 mg of protein per milliliter? Did higher or lower doses change your impression?

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IRSHAD CHAUDRY, MD, East Lansing, Mich: With your model you produced sepsis by a single puncture of a 25-gauge needle, and 50% of those animals survived. I wonder if you did an autopsy to find out whether the abscess was localized but in fact the animal had walled off and that was why 50% of them survived.

Is it possible that when you gave the wound fluid the abscess was not able to be contained and that may be the reason for the high mortality?

DR LAZAROU: I would like to thank the discussants for their questions. In response to Dr Simmons' question, the dose of wound fluid and serum that we used is the same as in previous in vivo studies with skin grafting. In that study we had administered wound fluid for 3 weeks and obtained 50% of those animals survived. I wonder if you had asked in your situation what happened because it contains high levels of asparaginase, we use fetal bovine serum for our thymic grafting. In that study we had administered wound fluid for 3 weeks and obtained 50% of those animals survived.