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Preliminary evidence of early bone resorption in a sheep model of acute burn injury: an observational study

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Abstract

Treatment with bisphosphonates within the first 10 days of severe burn injury completely prevents bone loss. We therefore postulated that bone resorption occurs early post burn and is the primary explanation for acute bone loss in these patients. Our objective was to assess bone for histological and biomechanical evidence of early resorption post burn. We designed a randomized controlled study utilizing a sheep model of burn injury. Three sheep received a 40 % total body surface area burn under isoflurane anesthesia, and three other sheep received cotton-smoke inhalation and

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served as control. Burned sheep were killed 5 days post procedure and controls were killed 2 days post procedure. Backscatter scanning electron microscopy was performed on iliac crests obtained immediately postmortem along with quantitative histomorphometry and compression testing to determine bone strength (Young's modulus). Blood ionized Ca was also determined in the first 24 h post procedure as was urinary CTx. Three of three sheep killed at 5 days had evidence of scalloping of the bone surface, an effect of bone resorption, whereas none of the three sheep killed at 2 days post procedure had scalloping. One of the three burned sheep killed at 5 days showed quantitative doubling of the eroded surface and halving of the bone volume compared to sham controls. Mean values of Young's modulus were approximately one third lower in the burned sheep killed at 5 days compared to controls, $p = 0.08$ by unpaired t test, suggesting weaker bone. These data suggest early post-burn bone resorption. Urine CTx normalized to creatinine did not differ between groups at 24 h post procedure because the large amounts of fluids received by the burned sheep may have diluted urine creatinine and CTx and because the urine volume produced by the burned sheep was threefold that of the controls. We calculated 24 h urinary CTx excretion, and with this calculation CTx excretion/24 h in the burned sheep was nearly twice that of the controls. Moreover, whole blood ionized Ca measured at 3- to 6-h intervals over the first 24 h in both burn and control sheep showed a 6 % reduction versus baseline in the burned sheep with <1 % reduction in the control animals. This sheep model was previously used to demonstrate upregulation of the parathyroid calcium-sensing receptor within the timeframe of the present study. Because both early bone resorption, supported by this study, and calcium-sensing receptor upregulation, consistent with the observed reduction in blood ionized Ca, are mediated by proinflammatory cytokines that are present as part of the post-burn systemic inflammatory response, we may postulate that post-burn upregulation of the parathyroid calcium-sensing receptor may be an adaptive response to clear the blood of excess calcium liberated by cytokine-mediated bone resorption.

Keywords

Burns; Calcium-sensing receptor; Sheep; Bone resorption; Inflammation

Introduction

Following severe burn injury, children develop hypocalcemic hypoparathyroidism within hours [1], lose 7 % of their lumbar spine bone mineral density (BMD) by 6 weeks post burn [2] and about 2–3 % of total body bone mineral content (BMC) by 6 months post burn [2]. By 2 weeks post-burn patients already have adynamic bone as evidenced by markedly reduced bone formation [3–5], reduced urinary markers of bone resorption [4], and reduced osteoblast differentiation [6]. However, if bisphosphonates are given intravenously within the first 10 days of the burn injury, the bone loss is entirely blocked [2, 5]. Therefore, it is possible that the acute bone loss could be the result of bone resorption. Given the rapid onset of the inflammatory response, which produces proinflammatory cytokines [4], and the acute stress response, which causes a three- to eightfold increase in endogenous glucocorticoids [4, 6], rapid bone resorption could result.

We therefore hypothesized that early bone resorption occurs before the onset of the steroid-induced osteoblast apoptosis, which would prevent the bone from compensating for the acute loss by new bone formation. Our aim was to assess bone resorption in an established sheep model of burn injury that was previously used to demonstrate upregulation of the parathyroid gland calcium-sensing receptor (CaSR) [7] as an explanation for the hypocalcemia, hypercalciuria, and hypoparathyroidism found acutely in burned children [1] and continuing up to 2 years [8]. Our intention, then, was to look at the sheep iliac crest for histological and biomechanical evidence of early bone resorption.

Materials and methods

Sheep

In total, three female Merino sheep weighing 30–40 kg underwent 40 % total body surface area flame burn under isoflurane anesthesia and three other sheep were sham operated, that is, intravenous catheters were inserted and isoflurane was administered, but no burn was carried out. The burn injury destroys the nerve endings in the skin and consequently the animals feel no pain. However, the animals received analgesics before and after the procedure: buprenorphine 0.01 mg/kg IM before and 0.01 mg/kg IV twice a day for 2 days after injury and then per need. Postoperatively they are mobile and eat and drink regularly without any impairment resulting from the burn. They were resuscitated by lactated Ringer's solution by formula: 4 ml/kg/percent burned surface for the first 24 h and 2 ml/kg/percent burned surface thereafter. The burned sheep killed at 5 days post procedure were not subjected to cotton-smoke inhalation.

Sheep were killed at either 2 days or 5 days post procedure. Controls available for this study consisted of sheep exposed to acute cotton-smoke inhalation, which is not known to adversely affect bone. These sheep can be maintained for only 2 days post cotton-smoke inhalation, so the initial time point of sacrifice was decided to be 2 days post burn. An initial study of bone histomorphometry of one sheep subject to both 40 % total body surface area burn and smoke inhalation and killed at 2 days post burn failed to demonstrate any differences from the control sheep (see Figs. 1 and 2 for comparison). Therefore, as we were able to maintain sheep with burn injury and no smoke inhalation for up to 5 days post burn, we chose to evaluate burn-only sheep at 5 days post burn, the limit to which sheep could be maintained as viable following burn injury. Iliac crests were removed at necropsy and 2 cm² samples immediately placed in 70 % ethanol. Samples were then processed for backscatter scanning electron microscopy with quantitative histomorphometry and biomechanical compression testing. In addition, whole blood was sampled for ionized Ca using a blood gas analyzer (Model GEM Premier 3000; Instrumental Laboratory, Lexington, KY, USA) at baseline and then at 3, 6, 12, 18, and 24 h post burn or sham burn; cumulative post-baseline data were converted into percent of baseline for each value. Mean and standard deviation (SD) were obtained for the burn and control groups separately. Urine samples were also analyzed at 24 h for C-telopeptide of type I collagen (CTX) using the CrossLaps assay (performed at the Mineral Metabolism Laboratory, Lurie Children's Hospital, Chicago, IL, USA). This study was approved by the Animal Care and Use Committee of the University

of Texas Medical Branch. All animals were handled according to the guidelines of the American Physiological Society and the National Institutes of Health.

Fixed samples were cut in 5-mm sections with a Buehler diamond saw (Buehler, Lake Bluff, IL, USA), trimmed to 0.8–1.2 cm in size, and embedded in methylmethacrylate (MMA). The plastic-embedded sections were ground by graded sandpaper [400, 800, or 1,200 grit (Buehler)] and the surface was then polished on an autogrinder/polisher by using Metadi diamond polishing compound (1 and 0.25 μm ; Buehler 40-6244 and 40-6112) for 1 min each. The samples were carbon coated and scanned with a FEI Quanta field emission gun environmental scanning electron microscope (FEG-ESEM) XL30. For the histomorphometry, we used AnalySIS Software (Soft Imaging Systems, Munster, Germany) measuring eroded surface by one to four nonadjacent 40 $\times$ field SEM pictures. Bone volume/total volume (BV/TV) was measured by whole area SEM pictures. The images from the 2-day sacrifice group were from the stereomicroscope.

For biomechanical testing, trabecular bone samples were prepared from each region using a low-speed diamond blade saw or sharp knife with continuous water cooling to produce a sample cube size of $6 \times 6 \times 6$ mm. Compression mechanical testing was performed in three directions in a MTS MiniBionix 858 (MTS, Minneapolis, MN, USA) axial load frame with a SMT2-200N load cell (Interface, Scottsdale, AZ, USA) and TestStar II control software. The loading protocol began with a 0.5 N preload to ensure full contact with all the interfaces and to eliminate effects caused by the surface condition of the sample. The loading then proceeded for 0.05 mm at a rate of 0.01 mm/s and retreated back to the post-preload position. This compressive force and the displacement of the loading piston were recorded and used for the calculation of the elastic modulus (Young's modulus) of the sample. The reported value of the elastic modulus is the averaged product of the values of all the tests and all the directions in each sample.

Results

Backscatter scanning electron microscopy and quantitative histomorphometry

All three burned sheep killed at 5 days post procedure exhibited scalloping of the bone surface, an effect of osteoclastic bone resorption. In contrast, none of the three sheep killed at 2 days post burn demonstrated scalloping (Fig. 1). Quantitative histomorphometry yielded variable results (Table 1), but one of the three sheep killed at 5 days demonstrated a doubling of eroded surface as a percentage of bone surface and a 50 % reduction in bone volume as a percentage of total volume compared to the sham controls. These data suggest that resorption may begin as early as 5 days post burn but may not become quantitatively distinct for a period of time that extends beyond the period during which we could sustain the sheep. Figure 2 shows the absence of scalloping in the initial iliac crest sample of a sheep killed 2 days post burn and cotton-smoke inhalation. This failure to demonstrate scalloping influenced us to study subsequent iliac crest specimens obtained 5 days post burn and in the absence of cotton-smoke inhalation.

Whole blood ionized calcium

When compared with baseline values, blood ionized calcium levels fell by 6 % in the burned sheep [93.9 ± 7 % (SD) of baseline ($n = 13$ samples in three sheep)], compared to 0.7 % [99.3 ± 6 % of baseline, in the sham controls ($n = 15$ samples in three sheep)] in the first 24 h of the study. Figure 3 shows the time-course of decrease in blood ionized calcium during the first 24 h post procedure in the burn and control sheep. Urine CTx at 24 h was not different between the burn and control groups: 8.1 ± 1.7 $\mu\text{g}/\text{mmol}$ creatinine versus 7.6 ± 1.4 $\mu\text{g}/\text{mmol}$ creatinine. However, because the burned sheep received large volumes of fluid during the first 24 h it is possible their urine was more diluted because urine creatinine concentration in the burned sheep was less than half that of controls, 3.2 ± 1.2 mM compared to 7.7 ± 4.3 mM. Furthermore, total urine volume in the 24 h post procedure was three times greater in the burned sheep than in the controls: 983 ± 95 ml/24 h compared to 313 ± 197 ml/24 h in the controls. Therefore, if total urinary CTx excretion per 24 h is examined, mean urine CTx excretion in the burned sheep is nearly twice that of controls, $25,298 \pm 15,579$ ng/24 h compared to $15,284 \pm 3,542$ ng/24 h in the controls.

Biomechanical compression testing

The mean and standard deviation of Young's modulus, a measure of compression strength, in the sheep killed at 5 days post burn was 29.8 ± 8 MPa, whereas in the sham controls killed at 2 days post procedure the mean was 37.7 ± 7 MPa (where 1 MPa is 1 million newtons/m²) by unpaired *t* test ($p = 0.08$) (Fig. 4). Although these numbers are not statistically significant they represent a trend toward weakening of the bone, which would be expected with evidence of early resorption.

Discussion

Our data provide evidence of bone resorption taking place as early as 5 days post burn. It is possible that if the three burned sheep were carried longer than 5 days we may have seen additional quantitative histomorphometric differences from the sham control sheep. However, this was not possible under present circumstances. Nevertheless, bone surface scalloping, a signature of resorption, was present in all three burned sheep and absent in the controls. Moreover, the trend toward biomechanically weaker bone as evidenced by the decline in Young's modulus is also consistent with early bone loss.

We recognize that the numbers of sheep studied were small and that the variability in quantitative histomorphometry and ionized Ca may be explained by the small sample size. Thus, the findings are therefore preliminary. However, the simultaneous trend toward mechanical weakness of the bone in the sheep killed at 5 days would also lend some support to the resorption evidence we present. The numbers of sheep studied were necessarily limited by cost, but we believe that the evidence is consistent with early resorption in the burned sheep killed at 5 days post burn, and a more extensive study is warranted to confirm these preliminary findings.

These early resorptive changes would explain the efficacy of acute intravenous administration of bisphosphonates in prevention of bone loss in children following burn

injury [2, 5]. In addition, a study in adults within the first week following burns by Leblebici et al. [9] demonstrated an elevation of urinary deoxypyridinoline excretion, also consistent with early increased resorption. The failure to demonstrate a significant difference between burn and control groups in urine CTx excretion when normalized to creatinine in the first 24 h may be caused by a difference in breeds. In an earlier study Chavassieux et al. [10] reported baseline urine CTx levels in sheep at ~400 µg/mmol creatinine, a value 100 fold greater than that observed in our study. The breed of sheep used in the older study is not specified but the large difference with our study results suggest that the CrossLaps assay is not sufficiently sensitive in the breed we used and therefore any real differences possibly could not be detected. Or, as indicated by the calculation of urinary CTx excretion per 24 h, differences in CTx excretion could have been swamped by the large amount of free water excreted following fluid resuscitation. In a 30-kg sheep, 4 ml/kg/day/percent burn injury is the equivalent of approximately 5 l extra fluid. Moreover, within the 2–5 day window of this study we previously demonstrated hypocalcemia in this same sheep model and a simultaneous 50 % upregulation of the parathyroid calcium-sensing receptor [7]. We also know that in addition to the presumptive acute bone resorption taking place in burned children, they also manifest the features of CaSR upregulation, namely, hypocalcemia, hypoparathyroidism, and urinary calcium wasting [1]. In the present study, the decline in post-baseline ionized calcium levels in the blood of the burned sheep but not in the control group is consistent with an upregulation of the CaSR, and the range of ionized calcium concentrations is similar to that previously reported [7].

As noted earlier, inflammatory cytokines cause bone resorption by stimulating osteoblasts to produce the ligand of the receptor activator of the nuclear transcription factor κ B (RANK ligand, or RANKL), which stimulates marrow stem cells to differentiate into osteoclasts and increase bone resorption. The cytokines also mediate CaSR upregulation [11–13]. Inasmuch as pediatric burn victims exhibit high circulating cytokine levels [4], it is likely that the proinflammatory cytokines mediate both resorption and CaSR upregulation in a similar time frame. Given that acute bone resorption leads to liberation of calcium from bone and its entry into the blood, we may speculate that the cytokine-mediated upregulation of the parathyroid calcium-sensing receptor may serve as an adaptive response that protects the body from resorption-induced hypercalcemia by suppressing parathyroid hormone secretion and permitting accelerated urinary calcium losses.

The clinical implications of this study include provision of the basis for the efficacy of early treatment with bisphosphonates to prevent post-burn bone loss and the postulation of a link between cytokine-mediated inflammatory bone loss and cytokine-mediated hypocalcemic hypoparathyroidism as a possible explanation for the significant calcium loss incurred by burn patients.

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Fig. 1. Scanning electron microscopic sections of iliac crest from sheep killed 5 days post burn (*top row*) and from three control sheep killed 2 days post procedure (*bottom*). Scalloping is clearly designated in the upper figures and not seen in the lower figures

2d post-burn

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Fig. 2. Scanning electron microscopic section of an iliac crest from a burned sheep killed 2 days post procedure and cotton-smoke inhalation. Scalloping is absent and the image is comparable to the control sheep seen in Fig. 1 (*bottom*)

Fig. 3. Mean whole blood ionized calcium concentration as a percentage of baseline levels (y-axis) shown over the first 24 h post procedure for the control sheep (*open bars*) and the burned sheep (*closed bars*)

Fig. 4.
Mean and standard deviations of mechanical modulus (Young's modulus) derived from compression testing of the sheep killed 5 days post burn and control sheep

Table 1

Quantitative histomorphometry of the iliac crest in burned and control sheep

	BV/TV (%)	ES/BS (%)
Burns ($n = 3$)	16.6 ± 5.5	16.9 ± 14.1
Burns ($n = 4$)	17.9 ± 5.2	17.8 ± 11.7
Controls ($n = 3$)	20.5 ± 1.5	15.5 ± 4.1

Burns ($n = 3$) indicates values for sheep killed 5 days post procedure

Burns ($n = 4$) indicates values for sheep killed 5 days post procedure plus the one sheep killed at 2 days post burn

BV/TV bone volume as a percentage of total volume of the specimen

ES/BS eroded surface as a percentage of bone surface of the specimen