Stimulation of Muscle Protein Degradation by Murine and Human Epidermal Cytokines: Relationship to Thermal Injury

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Accelerated muscle proteolysis is a characteristic of systemic reaction following trauma, sepsis, or extensive thermal injury. The factors involved in this accelerated muscle breakdown have not been fully described. However, recently leukocytic pyrogen or interleukin 1 (IL-1) have been implicated in the induction of muscle protein degradation in septicemia or trauma. The epidermal cytokine epidermal cell–derived thymocyte activating factor (ETAF) is biochemically and functionally similar to IL-1. Injury to skin can augment ETAF activity. Using a murine model, we found that thermal injury can significantly enhance ETAF/IL-1 activity in a dose-dependent fashion. In addition, ETAF can cause net muscle protein breakdown in vitro. Thus, increased amounts of ETAF produced by thermally injured skin may contribute to the accelerated muscle breakdown in extensive thermal injury. *J Invest Dermatol 87:711–714, 1986*

Skeletal muscle is the major protein reservoir in mammals. The balance between rates of protein synthesis and degradation in muscle is, therefore, an important determinant of body nitrogen balance. In muscle, rates of protein breakdown are precisely regulated by hormones and nutrients [1,2]. Accelerated muscle proteolysis is a characteristic of the systemic reaction following trauma, sepsis, and extensive thermal injury [3–5]. The consequences of an unbridled proteolytic state are often tragic, leading to sepsis and multisystem failure [6–8].

The factors that induce an acceleration in the breakdown of muscle protein following major burn injury have not been fully described. Clinical studies have established the central role of catecholamines in the "flow phase" post injury, and a substantial amount of information has been compiled concerning the role of insulin and certain other hormones on the relative rate of muscle protein breakdown [8–12]. More recently, other hormone-like substances have been discovered in critically ill patients that may exert additional metabolic effects [8,13]. Leukocytic pyrogen/interleukin 1 (IL-1) has been implicated in the induction of muscle protein degradation in patients with septicemia and ex-

tensive trauma [14,15]. The involvement of these factors in muscle proteolysis has further supported the role of the monocyte-macrophage in secretion of a group of regulatory proteins or monokines and their involvement in the metabolic responses to inflammation and sepsis.

In addition to monocyte-derived factors, it is now clear that epidermal cells can produce soluble factors or cytokines which are similar to monocyte-derived factors. One of these epidermal cytokines [16–19], epidermal cell thymocyte activating factor (ETAF), like IL-1 can mediate fever [18], cause leukocyte chemotaxis [18], augment interleukin-2 production by T cells [17], and cause T-cell chemotaxis [20], fibroblast and keratinocyte proliferation [19,21]. In addition, epidermal cytokine, perhaps distinct from ETAF, can regulate synthesis of major acute-phase plasma proteins in hepatic cells [19,22,23]. In this report we examined the effect of thermal injury on ETAF production. Using a murine model, we found that thermal injury significantly enhanced ETAF/IL-1 activity in a dose-dependent fashion. Because thermal injury enhanced ETAF/IL-1 activity and because extensive thermal injury is associated with significant muscle wasting, we investigated whether the epidermal cell-derived cytokine ETAF could induce net muscle protein breakdown.

MATERIALS AND METHODS

Cells Normal human epidermal cells were prepared from keratomed sections of whole skin by trypsin treatment, as previously described [17]. Murine epidermal cells were prepared as previously described [17]. Human or murine epidermal cells and the human skin carcinoma cell line COLO-16 were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) without any additional stimulants.

Preparation of Human Epidermal Supernatant Supernatant was collected after 5-day culture of subconfluent monolayers of COLO-16 cells or 24-h culture of normal human epidermal cells in DMEM containing 10% FCS. In order to measure ETAF

activity, the supernatant had to be dialyzed first for 24 h against phosphate-buffered saline (PBS) to remove inhibitory activities [17] and then was tested in the thymocyte costimulator assay. Results are expressed in units/ml as previously described [24]. On average, 1×10^6 COLO-16 cells release 100 units/24 h; 1×10^6 monocytes, 400 units; and 1×10^6 epidermal cells, 75 units. To obtain cytokines with higher specific activities, the media were concentrated approximately 10-fold by ultrafiltration using an Amicon ultrafiltration unit equipped with a YM-10 filter. A specific enrichment of thymocyte-stimulating activity was also achieved by gel filtration of 10-fold concentrated supernatant on a Sephadex G-100 column, using PBS as eluant. The fractions eluted from the column in the region of $M_r = 15,000$, and 4,000 were pooled and concentrated again by ultrafiltration. These were tested for proteolysis activity and ETAF activity.

Preparation of Murine-Derived Epidermal Cytokines Twenty-four hours after thermal injury mice were sacrificed and the injured skin was excised. The epidermis was separated from the dermis by trypsinization and an epidermal cell suspension was made as previously described [17]. The murine epidermal cells were adjusted to 1×10^6 epidermal cells/ml in DMEM containing 10% FCS. After 3 days, the cell-free supernatant was obtained and purified by ultrafiltration and Sephadex G-100 chromatography as above. Fractions containing high ETAF activity were pooled and aliquoted.

In other experiments, ETAF was further purified by sequential high-performance liquid chromatography (HPLC). Fractions eluted from the Sephadex column (15,000 M_r) were pooled and filtered through a 0.45-μm Millipore filter and chromatographed on a 41×250 mm Synchropak AX 300 ion exchange column (Synchrom Inc., Linden, Indiana), using a Gilson HPLC system. Starting buffer was 20 mM Tris-HCl at pH 7.6 and the gradient buffer was starting buffer augmented with 1 M NaCl. One-milliliter fractions were collected, dialyzed against PBS, and assayed in the thymocyte costimulator assay. Active fractions were then pooled and run on a TSK 3000 column using the above Gilson HPLC system. The active fractions corresponding to M_r 10,000–20,000 were pooled and tested in the thymocyte costimulator assay and in the proteolysis assay.

Method of Thermal Injury Female BALB/c mice, 8–10 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Maine) and used throughout the experiment. Sevitt has shown that the intensity of the lesion will vary with the temperature and the duration of heat application [25]. In our experiments the following temperatures were tested: 35°, 40°, 50°, 55°, 60°, and 65° centigrade. The duration of the burn in all cases was 30 s. This range of temperature allowed observations through a spectrum of mild, moderate, and severe thermal injury to skin. There were 6 mice in each temperature group. A 2 cm-diameter scald lesion was applied to the depilated dorsum of each animal using a sling-cradle template device which allowed immersion of the desired site into a temperature controlled water bath. Excellent anesthesia was provided using the ether bell-jar apparatus. Following the procedure, the mice were housed separately and fed water and standard chow ad libitum. The animals were sacrificed 24 h post burn and epidermal cell suspensions were made as described above.

Protein Breakdown The incubating medium for the rat muscle consisted of oxygenated Krebs-Henseleit bicarbonate buffer at pH 7.4, equilibrated with 95% oxygen and 5% carbon dioxide. The medium was supplemented with 5 mM glucose and 2% defatted bovine albumin, as well as with 19 amino acids at physiologic concentrations; however, no tyrosine was present in medium which included all other essential amino acids. Each muscle specimen (50–60 mg) was incubated for 2 h at 37°C. Net protein degradation was determined by measuring the rate of increase in the tyrosine concentration in the incubating medium at the beginning and end of the experiment, as previously described [15]. Data are reported as the bioactivity percent of control or as net protein degradation nmol/g/2 h.

Since the responses of muscles varied from rat to rat when incubated with a given fraction, it was deemed advisable to use as the control for each assay the soleus muscle of the opposite leg of each rat, incubated only in medium without test supernatant added. Each assay was carried out in quadruplicate with 4 pairs of muscles. The measured rates of net protein degradation in the 4 muscles (incubated with 0.25 ml of sample being tested) were averaged and compared with the average value for the 4 control muscles. Bioactivity of the solution being tested was expressed as the percentage of average net protein degradation in the untreated control muscles. The significance of differences between the mean values of net protein degradation induced by various samples was determined by Student's t-test.

Histology Histologic sections were prepared using the glycol methacrylate embedding procedure [26]. They were then stained using the modified Nocht's azure-eosin technique [27].

RESULTS

We have previously shown that both normal epidermal cells and the squamous cell carcinoma cell line constitutively produce ETAF [22]. Using normal or cell line-derived ETAF, we evaluated the ability of these cytokines to induce muscle proteolysis. As measured by the rate of release of amino acids, a significant elevation in net protein degradation was seen in muscle incubated with both normal epidermal cell-derived cytokines or COLO-16-derived cytokines. (Fig 1).

Effect of Thermal Injury on Skin Thermal injury to skin is capable of inducing significant morphologic and histologic changes, dependent on the temperature and the duration of thermal injury. In this study, no gross morphologic changes were seen in skin subjected to 55°C or less immediately postburn or 24 h postburn (Fig 2). However, some histologic changes were noted at 55°C postburn which revealed there was evidence of edema within the epidermis (spongiosis) with some pyknotic changes in the keratinocytes (Fig 3). In addition, the dermis appeared edematous and
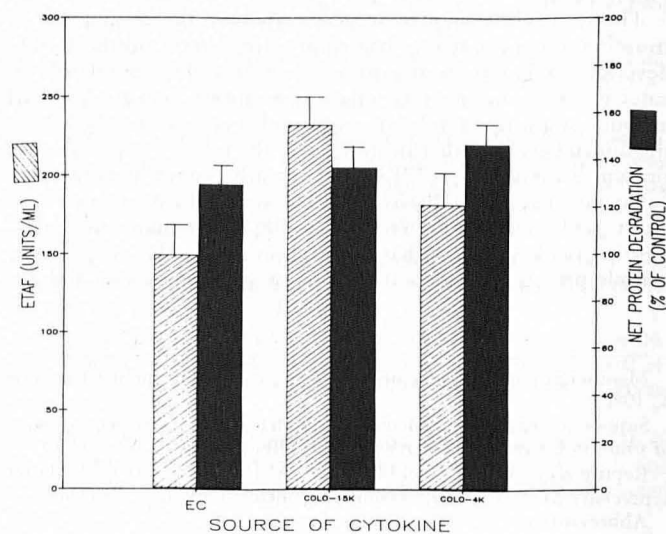
Figure 1. ETAF and proteolytic activity from normal epidermal cells and from a skin squamous cell carcinoma cell line. The ETAF activity was quantitated using the thymocyte costimulator assay. Supernatants from normal epidermal cells (*EC*) or from the skin squamous cell carcinoma cell line COLO-16 were purified by Sephadex G-100 chromatography. Fractions containing high ETAF activity (15,000 dalton for normal epidermal cells, [*EC*] and either 15,000 [*COLO-15K*] or 4,000 [*COLO-4K*] for the COLO 16 cell line) were tested for proteolytic activity. The above fractions were added to rat muscle for 2 h at 37°C. Net protein degradation was determined by measuring the rate of increase in tyrosine concentration in the incubating medium at the beginning and end of the experiment. Results are expressed as percent of control. Control cultures had inactive ETAF added. *Bars,* SE.
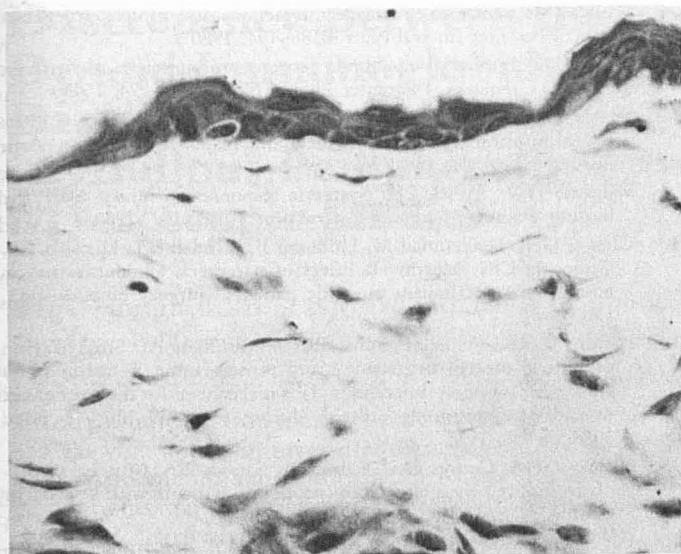
Figure 2. Histologic appearance of mouse skin 24 h after 30-s exposure to 45°C (× 500).

Figure 4. Histologic appearance of mouse skin 24 h after 30-s exposure to 60°C (× 500).
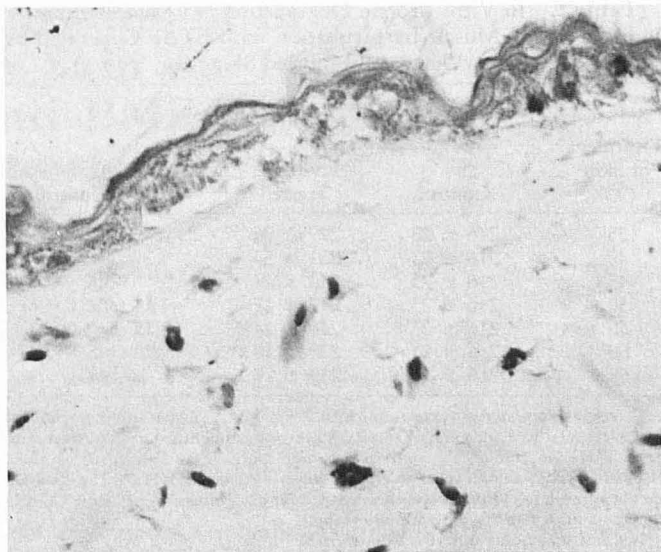
a mild inflammatory infiltrate was present. At 3 days, the 55°C burn showed areas of focal epidermal necrosis among the areas of viable tissue, suggesting that focal areas of the burn were severely damaged and converted to third-degree burns. At temperatures greater than 55°C (Fig 4), there was marked epidermal necrosis at 24 h.

Effect of Thermal Injury on ETAF/IL-1 Because of potential contaminating proteins in partially purified ETAF preparations, the supernatant generated from thermally injured skin was purified for ETAF activity using Sephadex G-100 chromatography followed by anion exchange HPLC and size exclusion HPLC columns. The resultant fraction (mean M_r 17,000) was then used for ETAF assay and proteolysis assay. Figure 5 illustrates the ETAF levels after thermal injury (mean ± SD) increase in ETAF/IL-1 after thermal injury. Peak activity is seen after exposure to 55°C.

Effect of Thermal Injury on Net Protein Degradation Factor Using highly purified samples from burned skin, we found significant net protein breakdown activity in the 50°C and 55°C

samples (Table I). The 55°C sample also corresponded to peak activity in thymocyte costimulator assay. Because these samples were diluted, column chromatography activity is somewhat lower than the activity found with the partially purified samples.

DISCUSSION

Local events in the burn wound are characterized by inflammation, while the systemic response following major thermal injury is characterized by shock and cardiovascular instability, followed by alterations in host defense mechanisms and an increased basal metabolic rate with extensive muscle wasting [28]. Evidence for a link between the injured peripheral tissue and the more generalized response has been strengthened by the established role of the monocyte-macrophage in the secretion of regulatory monokines and recently identified mechanisms relating IL-1 activity to metabolic adjustments in septic shock [14,15].

Using an in vivo murine model, we found that graded thermal injury (30-s exposure) significantly increased ETAF activity in a dose-dependent fashion, with peak activity at 55°C. This was

Figure 3. Histologic appearance of mouse skin 24 h after 30-s exposure to 55°C (× 500).
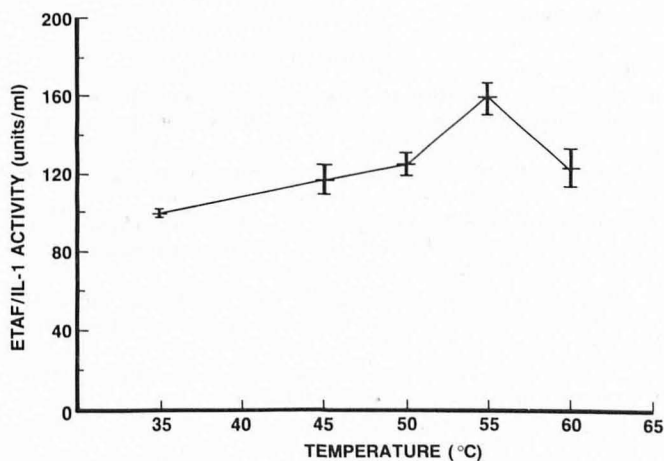
Figure 5. Effect of temperature on ETAF generation. Mice were exposed to temperatures from 35°–65°C. Twenty-hour hours later, skin was obtained and cultured as outlined in *Materials and Methods*. Supernatant from these cultures was sequentially purified and tested for ETAF activity in the thymocyte costimulator assay. *Bars*, SE.

Table I. In Vitro Protein Degradation (Tyrosine Release) Induced in Rat Muscle by Incubation with ETAF Generated by Thermally Injured Skin

Treatment of Skin (°C)	Muscle Proteolysis (ng tyrosine/g tissue/2 h)		Percent Difference from Control
	Control	Cytokine Treated	
35	216 ± 23	229 ± 35	NS[a]
40	216 ± 23	221 ± 54	NS
45	216 ± 23	210 ± 24	NS
50	216 ± 23	247 ± 27	+14 ($p < 0.025$)
55	216 ± 23	248 ± 24	+15 ($p < 0.05$)
60	216 ± 23	220 ± 18	NS
65	216 ± 23	215 ± 27	NS

Mice were subjected to temperatures from 35°–65°C. Twenty-four hours later mice were sacrificed and epidermal cell cultures were established from burned skin. After 3 days in culture, the supernatants were purified by sequential Sephadex chromatography, anion exchange HPLC, and size exclusion HPLC. The resultant fractions (mean M_r 17,000) were then assayed for net protein degradation. Control represents muscle incubated without cytokine.

[a] NS, not statistically different from control.

seen with crude supernatant generated from thermally injured mouse skin as well as from supernatant purified for ETAF activity by sequential HPLC. These results are consistent with the recent observation by Kupper et al [29], who found that in vitro heat treatment of keratinocytes led to enhanced ETAF production. Using our in vivo system, we found that activity decreased at higher temperatures, presumably due to decreased cell viability. Highly purified ETAF preparations derived from thermally injured mouse skin were also found to contain significant protein degradation activity.

Epidermal cytokines including ETAF are known to have a wide variety of effects on inflammatory and immune events. The ETAF-like IL-1 can mediate fever [18], cause leukocyte chemotaxis, both in vitro [18], and in vivo [30], augment interleukin-2 production by T cells [18], and cause fibroblast and keratinocyte proliferation [19,21,31]. In addition, epidermal cytokines, perhaps distinct from ETAF, can regulate synthesis of major acute-phase plasma proteins in hepatic cells [22,23]. Thus, ETAF, while produced locally in the skin, can mediate processes occurring at a considerable distance from the skin. In this study we have shown that ETAF production is augmented by thermal injury and that ETAF can induce net muscle protein degradation. It is possible, therefore, that increased local release of ETAF may play a significant role in the accelerated muscle protein breakdown seen in extensive thermal injury.

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