Overview of the Role for Calreticulin in the Enhancement of Wound Healing through Multiple Biological Effects

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Calreticulin (CRT), an intracellular chaperone protein crucial for the proper folding and transport of proteins through the endoplasmic reticulum, has more recent acclaim as a critical regulator of extracellular functions, particularly in mediating cellular migration and as a requirement for phagocytosis of apoptotic cells. Consistent with these functions, we show that the topical application of CRT has profound effects on the process of wound healing by causing a dose-dependent increase in epithelial migration and granulation tissue formation in both murine and porcine normal and impaired animal models of skin injury. These effects of CRT are substantiated, *in vitro*, as we show that CRT strongly induces cell migration/wound closure of human keratinocytes and fibroblasts, using a wound/scratch plate assay, and stimulates cellular proliferation of human keratinocytes, fibroblasts, and vascular endothelial cells, providing mechanistic insight into how CRT functions in repair. Similarly, in both animal models, the histology of the wounds show marked proliferation of basal keratinocytes and dermal fibroblasts, dense cellularity of the dermis with notably increased numbers of macrophages and well-organized collagen fibril deposition. Thus, CRT profoundly affects the wound healing process by recruiting cells essential for repair into the wound, stimulating cell growth, and increasing extracellular matrix production.

Journal of Investigative Dermatology Symposium Proceedings (2006) **11**, 57–65. doi:10.1038/sj.jidsymp.5650011

OVERVIEW OF CALRETICULIN INTRACELLULAR AND EXTRACELLULAR FUNCTIONS

Calreticulin (CRT), a Ca-binding protein, provides the important function of chaperoning glycoproteins through the endoplasmic reticulum (ER), ensuring proper protein folding and preventing aggregation (Krause and Michalak, 1997; Johnson *et al.*, 2001; Bedard *et al.*, 2005). A lectin site on CRT recognizes N-linked oligosaccharide processing intermediates of glycoproteins and prolonged interaction with misfolded proteins initiates rejection and subsequent direction to the proteasome for degradation. CRT also engages in direct protein-protein interactions. Another major function of CRT is to maintain intracellular calcium homeostasis (Arnaudeau *et al.*, 2002), important in signaling, particularly of integrins, which require calcium for protein interactions and functions. In this regard, CRT specifically chaperones alpha integrins through the ER and the binding of CRT to their cytoplasmic domains is tightly regulated by intracellular calcium levels required for integrin signaling, important in regulating cell shape, adhesion, spreading, and motility (Coppolino *et al.*, 1997; Kwon *et al.*, 2000). Transcriptional activation of the CRT gene, itself, is regulated by the modulation of cytosolic calcium concentration (Krause and Michalak, 1997). In addition, CRT has been shown to affect cell adhesion through calmodulin and calcium-mediated kinase (Bedard *et al.*, 2005). As the ER is contiguous with the nuclear membrane, which also stores calcium, CRT has been shown to be involved in nuclear functions including gene transcription and nucleocytoplasmic transport, particularly of p53 and myocyte enhancer factor-2 (Lynch *et al.*, 2005), associated with a decrease in apoptosis (Ashby and Tepikin, 2001). Interestingly, CRT directly binds to the identical amino-acid sequence, GFFKR, in the alpha integrin cytoplasmic tails as in the DNA-binding region of many

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Abbreviations: CD, cluster domain; CRT, calreticulin; EGF, epidermal growth factor; ER, endoplasmic reticulum; LRP, low-density lipoprotein receptor-related protein/CD91/α2-macroglobulin; TSP-1, thrombospondin-1; VEGF, vascular endothelial cell growth factor

Received 15 December 2005; accepted 14 March 2006

steroid receptors, to inhibit steroid-sensitive gene activation, as a major nuclear function of this protein (Burns *et al.*, 1994a, b; Dedhar, 1994; Dedhar *et al.*, 1994; Michalak *et al.*, 1996).

CRT is a ubiquitous cellular protein and its importance in cell function is underscored by the fact that CRT cDNA is conserved in most organisms, that there is 90% amino-acid homology among mammals, and that CRT null mice die mid-gestation, notably with severe cardiac abnormalities (Mesaeli *et al.*, 1999; Rauch *et al.*, 2000; Johnson *et al.*, 2001). CRT is a 46 kDa glycoprotein that is divided into three domains, preceded by an amino-terminal hydrophobic (ER) signal sequence. The P-domain contains the lectin site and a high-affinity Ca-binding region. The acidic carboxy-terminal C-domain contains the high-capacity, low-affinity Ca^{2+}, binding sequence and terminates in a KDEL (lysine, aspartic acid, glutamic acid, leucine) sequence for ER retrieval (Krause and Michalak, 1997; Bedard *et al.*, 2005). Most recently, for the first time, CRT was shown to exist outside the ER by retrotranslocation to the cytoplasm (Afshar *et al.*, 2005). However, despite a major effort to determine how CRT is transported out of the cell, to date, this mystery has not been solved. Although CRT has been found in the Golgi and may exit the cell passively by binding to other proteins, this is still only speculative. Nonetheless, a multitude of extracellular functions have been ascribed to CRT and it has been localized to the cell surface of many mammalian cell types (Johnson *et al.*, 2001; Bedard *et al.*, 2005).

The overexpression of CRT causes an increase in fibronectin and cell spreading as well as an upregulation of the cytoskeletal protein, vinculin (mRNA and protein), in focal adhesion contacts, all contributing to increased adhesion. Whereas the adhesion function is believed to be regulated by CRT from within the ER (Opas *et al.*, 1996), other functions important in both migration and phagocytosis, hinge on staunchly supported evidence for extracellular functions of CRT (Goicoechea *et al.*, 2000, 2002; Murphy-Ullrich, 2001; Orr *et al.*, 2002, 2003a, b; Gardai *et al.*, 2005). In fact, CRT is considered to be a cell surface receptor for thrombspondin-1 (TSP-1) on bovine arterial endothelial cells (Goicoechea *et al.*, 2000) and for complement component C1q (McCreal and Gasque, 2002; Ghiran *et al.*, 2003). Further, cell surface CRT binds to the carbohydrate constituent (mannose) of the cell adhesion and basement membrane protein, laminin, important in cell migration through integrin binding (White *et al.*, 1995; McDonnell *et al.*, 1996).

Importantly, a series of studies has shown that cell surface CRT interacts with the heparin-binding domain I of TSP-1 to mediate focal adhesion disassembly, for migration. The binding site in the N-terminus of CRT that binds heparin-binding domain I, to mediate TSP-1 signaling, has been localized to amino-acid residues 19–36, and accordingly, this peptide blocks focal adhesion disassembly in fibroblasts and endothelial cells (Goicoechea *et al.*, 2002). Phosphatidylinositol 3-kinase is activated during the interaction of CRT with TSP as well as guanine nucleotide protein and extracellular signal-regulated kinase (phosphorylation) pathways to promote the cytoskeletal changes associated with this process of de-adhesion. This is termed an "intermediate adhesive state" that aids in the ability of cells to respond to microenvironmental stimuli (Murphy-Ullrich, 2001; Orr *et al.*, 2002, 2003a). CRT null mouse embryo fibroblasts do not respond to TSP-1/heparin-binding domain I, but can be rescued with exogenous CRT (Goicoechea *et al.*, 2002). As cell surface CRT, as a receptor lacks a transmembrane domain, a search for a co-receptor that might mediate its action revealed an interaction between CRT and the "promiscuous" low-density lipoprotein receptor-related protein (LRP/cluster domain (CD)91/α2-macroglobulin) (Orr *et al.*, 2003a). Finally, the role for the TSP-1/CRT/LRP receptor in complex-induced focal adhesion disassembly, as a component necessary for cell migration, was elegantly illustrated using both CRT and LRP null mouse embryo fibroblasts that failed to migrate in response to TSP-1 (Orr *et al.*, 2003a).

LRP interacts with CRT on the same cell (*cis*-interaction) to enable cellular migration. Most recently, it has been shown that the LRP on phagocytic cells is activated by interacting with CRT and phosphotidylserine on the surface of apoptotic cells (*trans*-interaction), as a default signal for their engulfment by both professional and non-professional phagocytes, such as fibroblasts (Gardai *et al.*, 2005). This discovery of CRT as a "universal" mediator of apoptotic cell clearance was prompted by CRT-deficient dead cells that were not phagocytosed by macrophages without the addition of exogenously supplied CRT. In these studies, the activation of LRP by soluble CRT stimulates micropinocytosis, activates rac-1, and induces the engulfment of cells passively bound by the phagocyte. Although CRT is on the surface of both live and dead cells, the presence of CD47 (integrin-associated protein (IAP)) blocks the uptake of live cells by phagocytes. Therefore, the downregulation of CD47 is essential for CRT to activate LRP on the phagocyte to signal uptake of dead cells, and accordingly, live cells are engulfed by simply blocking CD47. This function of CRT has obvious beneficial application in wound healing for debriding injured tissue and studies are underway to determine whether CRT may, in addition, be required or at least enhance the clearance of bacterial contaminants of the wound bed. As another function, CRT has been shown to exert a protective effect on damaged blood vessels through the C-domain (Johnson *et al.*, 2001). Therefore, one can ascertain that these extracellular activities are consistent with a role for CRT in wound repair, and importantly, provide mechanistic insight of its action in this process.

CHARACTERIZATION OF CRT AS THE ACTIVE COMPONENT IN WOUND HEALING OF A HYALURONAN-RICH ISOLATE FROM FETAL SHEEP SKIN

With the background knowledge of CRT, predominantly as an intracellular protein with no evidence for secretion, our rationale for the use of this protein in wound healing studies deserves explanation. In previous studies, it was shown that hyaluronic acid (HA) isolated from fetal sheep accelerated repair in a rat animal model. However, the beneficial effects were obviated by heat and protease treatment but not by hyaluronidase (Burd *et al.*, 1991). The active protein was termed hyaluronan protein complex (HA-PC) (Bakshandeh

et al., 1992; Cabrera *et al.*, 1995), and subsequently, following purification and amino-acid sequencing, the active protein component of the HA isolate was determined to be CRT. In the following wound healing studies described herein, a recombinant his-tagged protein produced in *E. coli* (Baksh *et al.*, 1992), as described, was used. Our approach involved the use of murine and porcine models of wound repair as well as *in vitro* studies that investigated the effects of CRT on proliferation and migration of human keratinocytes and fibroblasts. As this overview is a report from a meeting presentation, the experimental detail will be described briefly.

CRT INCREASES THE RATE AND QUALITY OF WOUND HEALING *IN VIVO*

Although loose-skinned hairy animals such as rodents offer good animal model systems to study wound healing for many reasons, including large trial numbers, they do not heal like humans as the panniculus carnosus muscle directly below the skin causes contraction preventing the evaluation of wound resurfacing or re-epithelialization. In contrast, pigs heal more like humans for many reasons including healing by epithelial migration over the wound bed, the concentration of hair follicles, and the presence of sweat glands (Bennett *et al.*, 2001; Sullivan *et al.*, 2001). Therefore, we chose to determine the effects of CRT on wound healing in both animal models, comparing the effects of CRT with the positive controls, vascular endothelial cell growth factor (VEGF), an angiogenic growth factor and platelet-derived growth factor (PDGF; BB chain), a growth factor for fibroblasts and endothelial cells (in a formulation/vehicle known as Becaplermin or Regranex gel) (Embil *et al.*, 2000; Nagai and Embil, 2002), in the murine and porcine models, respectively. Both factors have beneficial effects on wound repair by (only) targeting the dermis through increasing granulation tissue formation (Embil *et al.*, 2000; Nagai and Embil, 2002; Galiano *et al.*, 2004b; Michaels *et al.*, 2005). The extent of granulation tissue formation results from recruitment of cells into a wound, proliferation of the cells that inhabit the newly forming dermis, and production of extracellular matrix proteins by the composite cells. Re-epithelialization of the wounds, resulting from epithelial migration, is aided by an abundant provisional matrix/granulation tissue. As CRT is a calcium-binding protein, we reasoned that the appropriate conformation of the molecule for biological activity might require calcium. Wounds treated with CRT in buffer (10 mM Tris) without or with calcium (3.0 mM) showed no statistically significant difference in any parameters measured for healing. Therefore, the data reported herein used buffers containing calcium. The protocols for both the murine and porcine studies were approved by the Institutional Animal and Use Committee (IACUC) of New York University School of Medicine and Vanderbilt University Medical Center, respectively.

Murine studies

An increased effect on wound healing by any agent has rarely been observed in the normal mouse. Therefore, we used 8 to 12-week-old *db/db* mice (BKS.Cg-m + /lepr^{db}—; leptin receptor-deficient model of type II diabetes mellitus) that have impaired wound healing (Coleman, 1982). Normal C57BL/6J mice of the same age were also tested. A novel improved method that more closely simulates human healing by minimizing wound contraction in the mouse was used, as described (Galiano *et al.*, 2004a). Briefly, a circular donut-shaped splint centered (shown as orange surrounding the wound in Figure 1) over the wound permits healing by epithelial migration over the newly produced provisional matrix to close the wound gap. The splint allows direct application of the CRT and visibility for digital photographs used to measure wound closure. CRT at 1.0 and 5.0 mg/ml in 10 mM Tris containing 3 mM Ca, buffer alone, phosphate-buffered saline, and VEGF (1.0 mg/ml) in a volume of 0.01 ml were separately applied to two 5-mm-diameter full-thickness excisional wounds at the onset of wounding and everyday thereafter for 4 days (Galiano *et al.*, 2004b; Michaels *et al.*, 2005). The wounds were measured daily and days to full closure determined. Time to closure is defined as the time (day) the wound bed is completely closed/filled and epithelial gap is defined as the area of the wound uncovered by migrating epithelia (note: a wound is re-epithelialized before it is completely closed). The closure of the wounds, epithelial gap, and granulation tissue (newly formed dermis) were measured morphometrically and the analysis of wound measurements and statistics were performed, as described (Michaels *et al.*, 2005). To measure cell proliferation *in vivo*, mice were injected with bromodeoxyuridine at 4 hours before harvesting the wounds. As expected, there was no difference in any parameters of healing measured among CRT, buffer, or VEGF treatments in normal mice. However, using the *db/db* impaired model of wound repair, the CRT (5.0 mg/ml)-treated wounds showed a statistically significant difference in the extent of wound closure, measured as percent of the original size, from day 14 onwards, compared to control wounds, as shown in Table 1. The CRT-treated

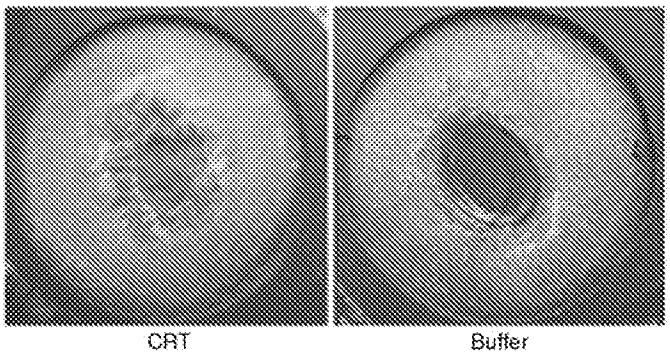

Figure 1. Comparison of gross wound closure in a (left) CRT (5.0 mg/ml)-treated mouse wounds *versus* (right) buffer control at day 14. Wounding of *db/db* mice is described in the text. The orange background surrounding the wound depicts the silicon splint centered over the 5.0 mm diameter full-thickness excisional wound to prevent wound contraction. CRT is diluted in buffer containing 10 mM Tris, 3 mM Ca (pH 7.0). Wound closure is shown to be greater in the CRT-treated wound ($P \leq 0.05$).

Table 1. Days to closure

	Buffer	CRT	
Day 0	1.00±0.00	1.00±0.00	—
Day 2	0.95±0.04	0.89±0.08	—
Day 4	0.86±0.08	0.76±0.07	—
Day 6	0.74±0.05	0.65±0.09	—
Day 8	0.61±0.03	0.51±0.08	—
Day 10	0.47±0.10	0.38±0.20	—
Day 12	0.43±0.09	0.27±0.17	—
Day 14	0.32±0.22	0.11±0.10	*$P<0.05$
Day 16	0.29±0.22	0.06±0.06	*$P<0.05$
Day 18	0.26±0.21	0.03±0.04	*$P<0.05$
Day 20	0.22±0.21	0.00±0.00	*$P<0.05$
Day 22	0.21±0.20	0.00±0.00	*$P<0.05$
Day 24	0.04±0.06	0.00±0.00	—
Day 26	0.00±0.00	0.00±0.00	—

CRT=Calreticulin.
Kinetics of wound closure in CRT (5.0 mg/ml)-treated wounds *versus* buffer controls. Mouse wounds were photographed every other day and open areas were calculated morphometrically, as described (Galiano *et al.*, 2004a; Michaels *et al.*, 2005). The data represent the mean of the percent remaining open compared to the area of the original wound size±SE (area at day 0=100%) of wounds *db/db* mice. Differences in average wound size between the groups was statistically significant ($P<0.05$) from days 14 to 22. The average time for complete wound closure in the CRT-treated wounds was 17.60 compared to 23.2 days in the buffer-treated controls ($P\leq0.045$, $n=5$ at each time point). A two-tailed Student's *t*-test was used to compute the *P*-value.

Table 2. Depth of granulation tissue

	Buffer	CRT
Day 3	25,922±8,591	82,115±58,772
Day 7*	29,208±5,366	50,742±9,272
Day 14	60,199±2,3982	97,269±63,817

CRT=Calreticulin.
*$P<0.001$.
Granulation tissue formation in CRT (5.0 mg/ml)-treated wounds *versus* buffer controls. Wounds were harvested at days 3, 7, and 14 and 5.0 μm histological sections were photographed and analyzed using morphometric software, as described (Michaels *et al.*, 2005). Data represent the mean number of pixels±SE of granulation tissue present in wounds from between three and nine *db/db* mice for each day and treatment parameter. The increased amount of granulation tissue formation in CRT-treated wounds was statistically significant on day 7 ($P<0.001$). A two-tailed Student's *t*-test was used to compute the *P*-value.

mice showed average complete wound closure at 17.60 days post-wounding compared to the control wounds with an average of 23.2 days to closure ($P\leq0.045$; $n=5$ at each time point). Figure 1 shows a representative comparison between a CRT and buffer-treated wound at 14 days post-wounding. Similarly, CRT induced a statistically significant difference in extent of epithelial migration over the wound (epithelial gap measurements) at day 7 ($P\leq0.039$; $n=6$), but not at day 3 post-injury ($n=6$); by day 14, the epithelial gaps were closed. Furthermore, CRT induced a greater area of granulation tissue beneath the wounds, which was statistically significant at the higher dose at day 7 post-wounding in one set of experiments (Table 2; $P\leq0.001$; $n=9$ wounds) and at day 14 in another set of experiments.

Using picrosirius red staining for collagen, Figure 2 shows the dose-dependent increase in granulation tissue at 1.0 and 5.0 mg/ml compared to the buffer control and VEGF (described below). Whereas these experiments are preliminary (unpublished) and ongoing, the data show that CRT, using 200 μg/mouse, over 4 days, showed positive effects on improving both the rate and quality of wound healing in an impaired murine model. The results using VEGF have been reported elsewhere (Galiano *et al.*, 2004b; Michaels *et al.*, 2005). These results suggest potential effects of CRT on migration and proliferation of cells involved in repair of the damaged epidermis and dermis in a diabetic wound healing model.

Porcine studies

These studies were performed on six Yorkshire pigs; three normal and three rendered steroid impaired by injection of methylprednisolone 2 days before wounding. Four longitudinal partial thickness wounds, removing only part of the dermis (1560 μm deep) separated by pieces of intact skin secured with sutures (as bridges between wounds) were created on the dorsum of the pigs, as described (Bennett *et al.*, 2001). The wounds were treated with CRT (1.0–5.0 mg/ml), buffer, or Regranex (0.01%) at the onset of wounding, and then treated for three more successive days (CRT: 100 and 500 μg total per pig). Wounds for harvesting at 10 days (eight wounds) were made first and another set (eight wounds) was made 5 days later. In the normal pigs, re-epithelialization was complete by 7 days post-wounding. As in the human, keratinocytes that re-epithelialize the wounds migrate from both the wound margins and hair follicles, creating islands of epithelialized surface. The total coverage of the wound resulting from both epithelial migration and proliferation was quantitated morphometrically (Image Pro Plus) and expressed as percent healed. Data were expressed as mean±SE and statistical significance was determined by analysis of variance and Bonferroni's *post hoc* test. Short of reaching statistical significance, at day 5 after wounding, the CRT-treated wounds appeared more mature showing greater stratification compared to Regranex (percent healed: 1.0 mg/ml CRT=58% *versus* Regranex=40%). Importantly, whereas the CRT-treated wounds were completely resurfaced by 10 days, the Regranex-treated wounds were not re-epithelialized at this time point ($n=6$ wounds per category). Granulation tissue formation, measured morphometrically as dermal depth (distance between the newly formed dermis, from the junction of the epidermis extending to the dermis that was left unwounded), showed a statistically significant increase in the CRT (5.0 mg/ml)-treated wounds compared to Regranex at 5 days post-wounding ($P\leq0.04$; $n=6$). Moreover, a dose response in the CRT-treated wounds (1.0 *versus* 5.0 mg/ml) was obtained indicating the specificity of the response ($P\leq0.058$; $n=6$). It was noted that the steroid-impaired pigs showed varied inherent differences in healing.

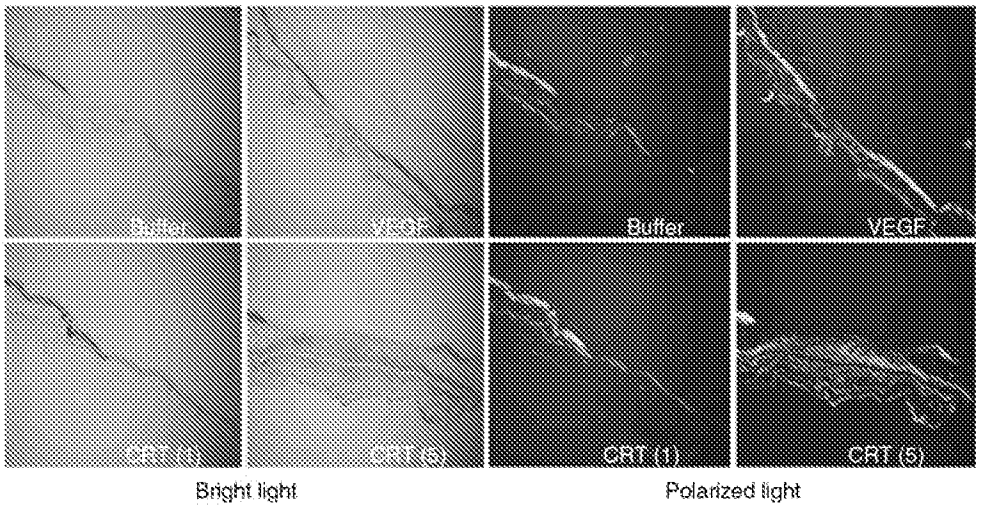

Figure 2 Collagen deposition by picrosirius red staining of CRT (1.0 mg/ml; 5.0 mg/ml), VEGF (1.0 mg/ml), and buffer-treated mouse wounds at 10 days after injury. Slides containing mouse tissue were stained with picrosirius red, as described (Noorlander *et al.*, 2002; Cuttle *et al.*, 2005) and examined by bright field (left panels) and polarized light (right panels). Under polarized light, yellow-green staining denotes a more-organized and less crosslinked collagen and a yellow-red hue indicates more highly crosslinked collagen. Both the panels depicting wounds, under bright field and polarized light, show a dose-dependent increase in collagen deposition in the area of granulation tissue in the CRT-treated wounds. The yellow-green birefringence shown in the 5.0 mg/ml (5) CRT-treated wound (lower right panel) suggests a more organized and less crosslinked collagen matrix compared to the VEGF-treated wounds (upper right panel). The increase in granulation tissue is owing to both the abundant cellularity and matrix deposition in the CRT-treated wounds. Buffer, 0.01 M Tris, 3.0 mM calcium, (pH 7.0); CRT (1), CRT in buffer at 1.0 mg/ml; and CRT (5), CRT in buffer at 5.0 mg/ml.

The differences in percent healed and depth of granulation tissue of these wounds harvested at 6 days post-wounding was unremarkable ($n = 6$–8 per category), except a more dense/compacted granulation tissue was noted in the CRT-treated wounds, possibly indicating that these wounds were more mature.

Both the murine and porcine studies provided evidence that CRT had positive effects on wound healing equal to or better than the chosen positive controls. However, the histology of the wounds showed remarkably unique characteristics in the CRT-treated wounds indicative of a more profound effect on wound healing.

HISTOLOGICAL ANALYSES OF THE WOUNDS IMPLICATE MARKED BIOLOGICAL EFFECTS OF CRT ON WOUND HEALING

The histology of the wounds was highly impressive, and importantly, the murine and porcine models showed similar unique characteristics in the CRT-treated wounds. Evidence for a strong effect and dose-dependent effect of CRT on both the epidermis and dermis consistent with improved wound repair compared to the negative controls was shown in both animal models. Furthermore, as described below, CRT induced markedly different effects on aspects of wound healing and, particularly, in cells involved in remodeling compared to the positive controls, VEGF (mouse) and PDGF (pig).

CRT strongly and dose-dependently induces cellular proliferation in basal keratinocytes and dermal cells

The hematoxylin–eosin-stained wounds revealed a dose-dependent increase in cellularity in the remodeling wound bed in the CRT-treated murine wounds, particularly on days 3–14, which was greater than the VEGF-treated wounds. This high degree of cellularity was contributed by a higher rate of proliferation of the cells of the entire wound bed observed following immunostaining for bromodeoxyuridine in wounds harvested at 3 days post-wounding.

This greatly increased cellularity of the dermis was also shown in the normal and impaired CRT-treated porcine wounds. Interestingly, there was a unique dose-dependent increase in kinetichore nuclear protein 67 (marker of proliferation) immunostaining that was localized to the nearly all basal and some suprabasal keratinocytes in both the normal porcine wounds, at 5 days, when an epithelial gap still remained open, and in the closed steroid-treated pigs at 7 days post-wounding. This specific pattern of kinetichore nuclear protein 67-positive cells was also observed in the unclosed murine wounds examined at 7 days after wounding. Moreover, there were many more proliferating cells in the dermis (appeared to be fibroblasts) in both the porcine and murine wound models treated with CRT than in either the negative or positive controls at all time points examined. As shown previously, the keratinocytes of the migrating epithelial tongue were not kinetichore nuclear protein 67-positive, as cells that migrate do not proliferate (Werner and Munz, 2000; Onuma *et al.*, 2001). The strong proliferative effect of CRT on the basal keratinocytes before and after re-epithelization may indicate that these cells continue to be involved in wound remodeling (Usui *et al.*, 2005), which we show herein is enhanced by CRT.

CRT-treated wounds had increased granulation tissue and evenly distributed collagen compared to the positive control

A dose-dependent increase in collagen deposition that was evenly dispersed within the highly cellular dermis as well as an increase in the area of granulation tissue (as shown in

Table 2) was evident in the CRT-treated murine wounds at all time points after 4 days by both trichrome and picrosirius red staining (Figure 2). Picrosirius red staining provides color-dependent information regarding collagen organization and maturity (Noorlander *et al.*, 2002; Cuttle *et al.*, 2005). As shown in Figure 2 (right panels) of wounds 10 days after injury, both CRT (5.0 mg/ml) and VEGF induced a robust deposition of collagen; a dose-dependent response is shown in the CRT-treated wounds. After day 7 and through day 14 post-injury, the collagen fibrils appeared more yellow-green in the CRT-treated wounds (bottom right panel), suggesting a better-organized and less crosslinked collagen matrix compared to a red-yellow pattern in the VEGF-treated wounds (upper right panel), which is consistent with higher cross-linking and potentially scarring. In addition, as a marker for granulation tissue formation, fibronectin was increased in the CRT-treated mouse and porcine wounds (data not shown). The increased dermal depth of the CRT-treated wounds, shown by both bright field (Figure 2, left) and polarized light (Figure 2, right), is owing to both increased cellularity and matrix deposition. The VEGF-treated wounds similarly show abundant collagen deposition, but were not as cellular as the CRT-treated wounds (not obvious with this staining technique). The possibility that CRT induced a better quality of healing as an example of an antiscarring effect deserves further investigation in appropriate models.

CRT uniquely induced an influx of macrophages into the wounds

The CRT-treated porcine wounds demonstrated a deluge of macrophages drawn into the wound bed, as determined by immunoreactivity using MAC387 antibody. By morphometric analysis of the non-impaired porcine wounds at 5 days after injury, a 3-fold increase in the total number of macrophages was observed throughout the wounds treated with both 1.0 and 5.0 mg/ml CRT compared to the control or Regranex ($P \leqslant 0.008$; $n = 6$–8). Interestingly, 50% more macrophages appeared to remain within the microvascular structures of the dermis than in the extracellular matrix. In the impaired model of wounding, this identical pattern was observed on day 6 post-injury wounds. We have not examined the effect of CRT on monocyte/macrophage migration *in vitro*. However, there is the possibility that the effect may be indirect through CRT-induced cytokine release by cells of the dermis including immune cells (Okuyama *et al.*, 2004). The uniquely interesting effect of macrophages sequestered in blood vessels is not understood at this time. Nonetheless, considering that CRT recently has the auspicious function as the "universal" mediator of phagocytosis of apoptotic cells (Gardai *et al.*, 2005), exogenous CRT in the treated wounds may have a 2-fold function: first, to attract and/or mediate the migration of macrophages and other phagocytes into the wound, and subsequently, to enhance their ability to debride the wound.

CRT is dynamically expressed after skin injury and throughout wound repair

Whether CRT plays a physiological role in wound healing would be substantiated by its dynamic expression during the process of skin repair after injury, which could putatively be enhanced with therapeutic applications of the protein. Using an antibody to CRT (from M. Michalak), similar temporal and spatial changes in the expression of CRT were observed during wound healing in both the porcine and murine wounds. Briefly, CRT immunoreactivity was decreased in the epidermis after wounding with no immunostaining in the migrating epithelium, which in the porcine wounds was evident in the keratinocytes migrating from the wound margins as well as those migrating up the hair follicles to re-epithelialize the wounds (data not shown). Interestingly, the basal keratinocytes in both the adjacent unwounded and wounded skin, including the hypertrophic epidermis of completely resurfaced wounds at 10 days post-wounding, expressed negligible amounts of CRT. As these are the same specific cells of the epidermis that proliferated in response to CRT, this result, although provocative, cannot be explained at this time. Thus, as basal keratinocytes respond to CRT, but do not appear to express the protein (or no longer detected by the anti-CRT antibody), this point may clearly represent an extracellular function for CRT, requiring specific receptor activation. CRT was greatly increased in the cells of the dermis in wounded compared to unwounded skin. Most notably, fibroblastic-type cells showed intense cytoplasmic immunoreactivity, and macrophages were notably highly immunoreactive whereas unwounded skin showed little dermal immunostaining. In light of these results, it will be interesting to determine the functional relevance of the expression of CRT in specific cell types in the wounds and whether they are intracellular- or extracellular-driven responses of CRT.

IN VITRO STUDIES RECAPITULATE THE WOUND HISTOLOGY AND PROVIDE MECHANISTIC INSIGHT INTO THE BIOLOGICAL EFFECTS OF CRT ON WOUND HEALING

CRT induces migration of human keratinocytes and human and mouse fibroblasts

Primary human keratinocytes from breast skin, kindly supplied by Marjana Tomic (Departments of Dermatology and Microbiology, NYU School of Medicine) and a human low passage dermal fibroblast cell line, CCD-1070Sks (ATTC) were used to interrogate whether epidermal and dermal cells could migrate in response to CRT, *in vitro*. The scratch plate assay is the standard and simple well-used *in vitro* model for wound re-epithelialization, which tests the function of migration, albeit, non-directed migration (Huang *et al.*, 1998; Lampugnani, 1999). Monolayers of keratinocytes or fibroblasts on plastic tissue culture plates were wounded, as described in Figure 3. The cells were treated with CRT in 3.0 mM calcium, and after 24 or 48 hours, the plates were photographed and percent wound closure determined, as described in Figure 3.

CRT (1–100 pg/ml) induced maximum wound closure of keratinocytes to 15% at 10 pg/ml in 48 hours compared to both buffer containing calcium and media, at 2% closure; epidermal growth factor (EGF) (10 ng/ml) used as a positive control induced 12% closure ($n = 5$). Calcium was shown to

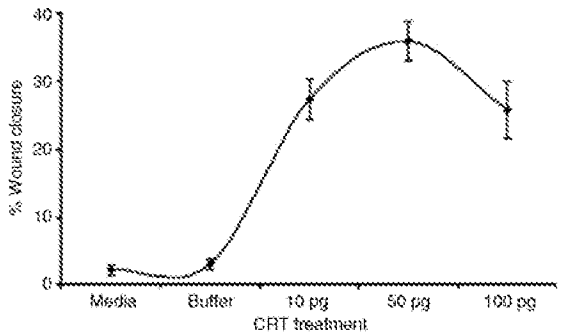

Figure 3. CRT induces migration of mouse fibroblasts using the scratch plate wound assay. Mouse 3T3 cells were seeded in 12-well tissue culture plates in DMEM, high glucose, and 10% calf serum. When the cells were 60–70% confluent, serum-free media was added, and after 16–24 hours, the cells were wounded using a 200 μl pipette tip to scrape and remove cells in a straight line through the center of the plate. The cells were treated with increasing concentrations of CRT, containing 3 mM calcium in media, and examined for migration over the wounded area at 24 and 48 hours. The plates were photographed and the area remaining uncovered by the cells at 48 hours was compared to the original free space (0 hour = 0% of the total area). The areas were traced and captured using Adobe Photoshop and the values obtained by National Institute of Health Image Analysis software were converted to percent wound closure. CRT without calcium induced minimal migration. All treatments were performed in duplicate ($n = 5$ experiments). The results showed that CRT maximally induces optimal wound closure of 36% at 50 pg/ml. EGF (10 ng/ml) was used as a positive control inducing 36% wound closure as well. The *x* axis is labeled in pg/ml.

be required to maintain the proper conformation of CRT for the function of inducing migration in both the fibroblasts and keratinocytes tested in our studies. We found that the effect of CRT, but not EGF, on keratinocyte migration was partially inhibited in the presence of mitomycin C. Therefore, the migration of keratinocytes in response to CRT may, in part, be related to CRT induction of proliferation as well as migration in keratinocytes. However, classic migration/chemotatic assays using transwells are necessary to verify this point.

CRT induced fibroblast migration with an optimal dose of 100 ng/ml, a 10,000 times higher dose than was required for keratinocytes. Whereas the keratinocytes appeared more sensitive, the fibroblasts migrated at a faster rate as they covered 23% of the wound in 24 hours compared to the buffer at 1.5% and EGF at 36% closure ($n = 10$). The 3T3 mouse embryo fibroblast cell line was more sensitive to CRT than the human fibroblasts as CRT induced maximal closure of 36% at 50 pg/ml compared to 2% and 36% (after 48 hours), in the buffer and mouse EGF control, respectively ($n = 5$; shown in Figure 3). A true migratory response to CRT was obtained with the fibroblasts as mitomycin C did not modulate migration. As TSP-1-induced migration is mediated by cell surface CRT (exogenously added or expressed by endothelial cells and fibroblasts) and the LRP (Goicoechea *et al.*, 2000; Orr *et al.*, 2002, 2003a, b; Barker *et al.*, 2004), we hypothesize that the cells that migrate into the wound may utilize focal adhesion disassembly as a mechanism of migration. Both the processes of migration and cellular proliferation likely contribute to the highly cellular granulation tissue we observed in the CRT-treated wounds. We presume that the abundance of these cells in the granulation tissue would positively impact the rate and quality of wound remodeling.

CRT induces proliferation of human keratinocytes and fibroblasts

The intense cellularity of the dermis and kinetichore nuclear protein 67 immunostaining in the keratinocytes and cells of the dermis suggested that CRT might directly induce cellular proliferation. The primary keratinocytes and fibroblast cell line described above were tested for their proliferative response to CRT using the CellTiter 96 assay (Promega, Madison, WI). CRT (0–200 pg/ml), diluted in buffer with or without 3 mM calcium, added to the cells (in triplicate) under serum-free conditions induced a 2.2-fold increase in keratinocytes proliferation at a maximum dose of 100 pg/ml after 72 hours, which was decreased at 200 pg/ml, giving a typical bell-shaped distribution ($n = 4$). For comparison, EGF (positive control; 10 ng/ml) stimulated proliferation to 1.3-fold over the untreated control. Similarly, the fibroblasts were induced to proliferate by CRT (0–150 ng/ml) with a maximal response (100 ng/ml CRT), which was 8.3-fold higher than the untreated controls ($n = 4$). EGF (10 ng/ml) induced a 7.8-fold increase in proliferation. Unlike the CRT-induced migratory/motility response in our experiments, calcium was not required for the proliferative response. This suggests that the Ca-dependent conformation of CRT may not be required for proliferation and that perhaps a different domain of the molecule may be involved. Whereas it has been shown that fibrinogen beta chain-induced fibroblast proliferation is mediated by cell surface CRT (Gray *et al.*, 1995), the effect of CRT on keratinocytes proliferation is an interesting and novel finding, opening a new area of investigation that undoubtedly will be relevant to many physiological processes. Potentially, functions may include the regulation of cell cycle proteins as CRT has been shown to function in the nucleus (Johnson *et al.*, 2001; Bedard *et al.*, 2005).

CRT AS AN AGENT FOR THE IMPROVEMENT OF (IMPAIRED) WOUND HEALING

The extent that CRT-mediated responses are directed from the ER or mediated extracellularly including different receptors and signaling intermediates, in the context of tissue repair, is an important future direction. The question of how CRT exits the cell to exert the positive effects on wound repair is an open question. Certainly, as injury to tissues induces cell death, CRT would be passively released as a normal requirement for wound repair. In addition, CRT is released from neutrophils upon their activation and from cytotoxic lymphocytes upon association with target cells (Burns *et al.*, 1992; Dupuis *et al.*, 1993; Andrin *et al.*, 1998; Cho *et al.*, 1999). Separately, it was also shown that CRT is a cell surface receptor on neutrophils that promotes signaling via a G-protein for activation of these cells, as evidenced by the generation of superoxide anion (Cho *et al.*, 1999). In this context, the oxidative stress associated with injury, likewise, causes mitochondrial release of reactive oxygen species and

ER stress, thereby causing CRT to upregulate the production of protective proteins (Bedard *et al.*, 2005). CRT has a profound effect on altering the total cellular levels of phosphotyrosine directed from within the ER (Burridge and Fath, 1989; Burridge *et al.*, 1992; Burridge and Chrzanowska-Wodnicka, 1996; Daniel and Reynolds, 1997; Schneider *et al.*, 1998; Fadel *et al.*, 1999, 2001; Burridge, 2005). As such, an effect on general signaling mechanisms involved in receptor activation for both migration/adhesion and proliferation may be important in the dynamic stochastic events required for wound repair.

We show that CRT exerts positive biological effects on both epidermal and dermal healing processes promoting an accelerated time to wound closure and an increased amount of highly cellular granulation tissue. Specifically, the *in vitro* and *in vivo* data purport effects of CRT on migration of cells into the wound, proliferation of cells to populate the wound, production of extracellular matrix for remodeling the wound, and evenly dispersed collagen to possibly lessen scarring. In addition, critical to wound repair, the removal of dead cells is a CRT-dependent process. This finding suggests a potential role for CRT in the uptake of bacteria to lessen infection. With this multifactorial effect of CRT on wound healing, we propose that CRT treatment may have therapeutic benefit to patients, such as diabetics with severe to delayed wound healing.

NOTE ADDED IN PROOF

Recent experiments show that human keratinocytes and fibroblasts migrated to a concentration gradient of calreticulin (CRT) using ChemoTx chambers (8.0 μm, membranes; Neuroprobe) with optimal doses of 10 pg/ml (18-fold increase over control) and 10 ng/ml (10-fold increase over control) in keratinocytes ($n = 3$) and fibroblasts ($n = 5$), respectively. These experiments show that CRT induces directed migration of human keratinocytes and fibroblasts. It is notable that similar optimal concentrations of CRT induced non-directed migration using the scratch plate assay, as described herein.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful for the support of these studies by Calretex Inc.,Tenafly, NJ. We thank Caprice Cadacio for help with this publication.

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