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Calpain inhibitors reduce the cornified cell envelope formation by inhibiting proteolytic processing of transglutaminase 1

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The abbreviations: CE, cornified cell envelope; NHEK, normal human epidermal keratinocytes; TGase(s), transglutaminase(s); TGase 1, the membrane-associated TGase or TGase K or Type I keratinocyte TGase; TGase 2, the ubiquitous tissue TGase or TGase C or Type II liver TGase; TGase 3, pro-TGase E or epidermal TGase

Abstract

Calpain I (μ-calpain) and II (m-calpain) are well known calcium-activated neutral cysteine proteases. Many reports have shown that activation of calpain is related to cataract formation, neuronal degeneration, blood clotting, ischemic injuries, muscular dystrophy and cornified cell envelope (CE) formation. Here, we report that insoluble CE formation was reduced after treatment with calpain I inhibitor (N-acetyl-leucyl-leucyl-norleucinal) on normal human epidermal keratinocytes (NHEK), whereas serine and thiol protease inhibitors had no effect on the reduction of CE. When NHEK cells were confluent, keratinocytes were treated with various concentrations (0.5 μM - 0.5 mM) of calpain I inhibitor or serine and thiol protease inhibitors under calcium induced differentiation. Insoluble CE formation was reduced about 90 % in the 50 μM calpain inhibitor I treated group by day 9 of culture, whereas insoluble CE was reduced only 10 % in the same condition. Interestingly TGase activity was blocked by 90 % in the 0.5 mM calpain inhibitor treated group within 72 h, whereas TGase activity was retained by 80 % in the 0.5 mM serine protease inhibitor treated group at 7 day treatment. Therefore it can be suggested that cysteine protease calpains might be responsible for the activation of the TGase 1 enzyme to complete insoluble CE formation during epidermal differentiation.

Keywords: calpain inhibitor, transglutaminase 1, cornified envelope

Introduction

Transglutaminase (TGase, EC 2.3.2.13) is a calcium dependent enzyme that catalyzes the formation of covalent ε-(γ-glutamyl)lysine cross-links in proteins (Folk and Finlayson, 1977). In the mammalian epidermis, there are three different TGases expressed. TGase 1 covers the most abundant messages among TGases in the epidermis and expression is observed from the basal layer to the top of the cornified envelope building a scaffold of barrier (Rice and Green, 1977; Thacher and Rice, 1985; Kim *et al*., 1994; Kim *et al*., 1995). We revealed that processing of TGase 1 is very specific, but by unknown protease(s) giving 20 times higher activity than intact form (Kim *et al*., 1995; Steinert *et al*., 1996). TGase 2 is an ubiquitous enzyme expressed in every cells and tissues (Ikura *et al*., 1988; Gentile *et al*., 1991). In the human epidermis, TGase 2 is expressed mostly in the supra basal layer and dermal layer which suggests it might be related with cell proliferation and extracellular matrix formation (Aeschilimann and Paulsson, 1991). Although many studies have revealed that TGase 2 can be related to apoptosis in neuronal tissues (Fesus *et al*., 1987), the physiological role of this enzyme is uncertain due to its ubiquitous expression and dual function as a GTPase enzyme (Nakaoka *et al*., 1994). Interestingly, only TGase 2 loses TGase activity among the TGase family by the action of proteases including calpain I (Zhang *et al*. 1998). This could be related with an *in vitro* experiment that demonstrated the increase of GTPase activity by specific truncation (Lai *et al*., 1996). TGase 3 is expressed on the upper granular layer to complete CE formation (Chung and Folk, 1972; Negi *et al*., 1985; Kim *et al*., 1990). Proteolysis of TGase 3 is involved in the active form which showed a 60 times higher activity than the pre-form (Kim *et al*., 1990). Though TGase 1 and 3 should require proteolysis for active catalysis, the specific protease which activates these TGases has not been reported to date. During terminal epidermal differentiation, proteolytic processing contributes to the increasing activity of epidermal transglutaminases (TGase 1 and 3) and also covalently cross-links precursor proteins to form CE in horney layers (Steinert and Marekov, 1995; Steinert *et al*., 1998). Noting this process, we explored the protease system which should be related to calcium dependent differentiation.

Calpain (EC 3.4.22.17) is a calcium dependent cysteine proteinase and ubiquitously distributed in various mammalian cells (Suzuki *et al*., 1987). Calpain forms a family consisting of at least six distinct members (Sorimachi *et al*., 1989, 1993, 1994). The family can be divided into

two groups on the basis of distribution - ubiquitous and tissue specific. However, our current knowledge of the biochemical properties of calpain applies mostly to the ubiquitous isozymes, calpain I (μ-calpain) and II (m-calpain). All the ubiquitous calpain isozymes are hetero dimers consisting of one large subunit and one small subunit. The small regulatory subunit carrying another calcium binding domain is common to both the isozymes (Sorimachi *et al*., 1994), though their amounts vary from one tissue to another. Both share similar biochemical characteristics except for the calcium concentration required for activation *in vitro*; calpain I is activated in the presence of micromolar levels of calcium whereas calpain II requires millimolar levels (Suzuki *et al*., 1987). Calpain I and II are known to be present in human skin and have been presumed to participate in various cellular functions mediated by calcium (Miyachi *et al*., 1986). One report demonstrated that platelet factor XIIIa (TGase) could be activated by calpain (Ando *et al*., 1987). However, there is no direct evidence for the contribution of calpains to processes of TGase 1 and 3 in the epidermis. Here we report that protease inhibitor studies revealed that calpain inhibitor specifically blocked the CE formation by inhibiting TGase 1 activation.

Materials and Methods

Conditions for TGase assays

A modified TGase assay method was used to determine enzyme activity by the measurement of the incorporation of [1,4^{14}C]-putrescine into succinylated casein (Folk and Chung, 1985). The samples were mixed in an reaction mixture (0.5 ml) containing 0.1 M Tris-acetate (pH 7.5), 1% succinylated casein, 1 mM EDTA, 10 mM $CaCl_2$, 0.5% lubrol PX, 5 mM DTT, 0.15 M NaCl (Sigma Chemical Co, St. Louis, MO, U.S.A.) and 0.5 mCi of [^{14}C]putrescine (118 Ci/mole) (Dupont-New England Nuclear, Wilmington, DE, U.S.A.). Following incubation at 37˚C for 1 h, the reaction was terminated by the addition of 4.5 ml of cold (4˚C) 7.5% TCA. The TCA-insoluble precipitates were collected on GF/A glass fiber filters (Whatman, England) washed with cold 5% TCA, dried and then counted.

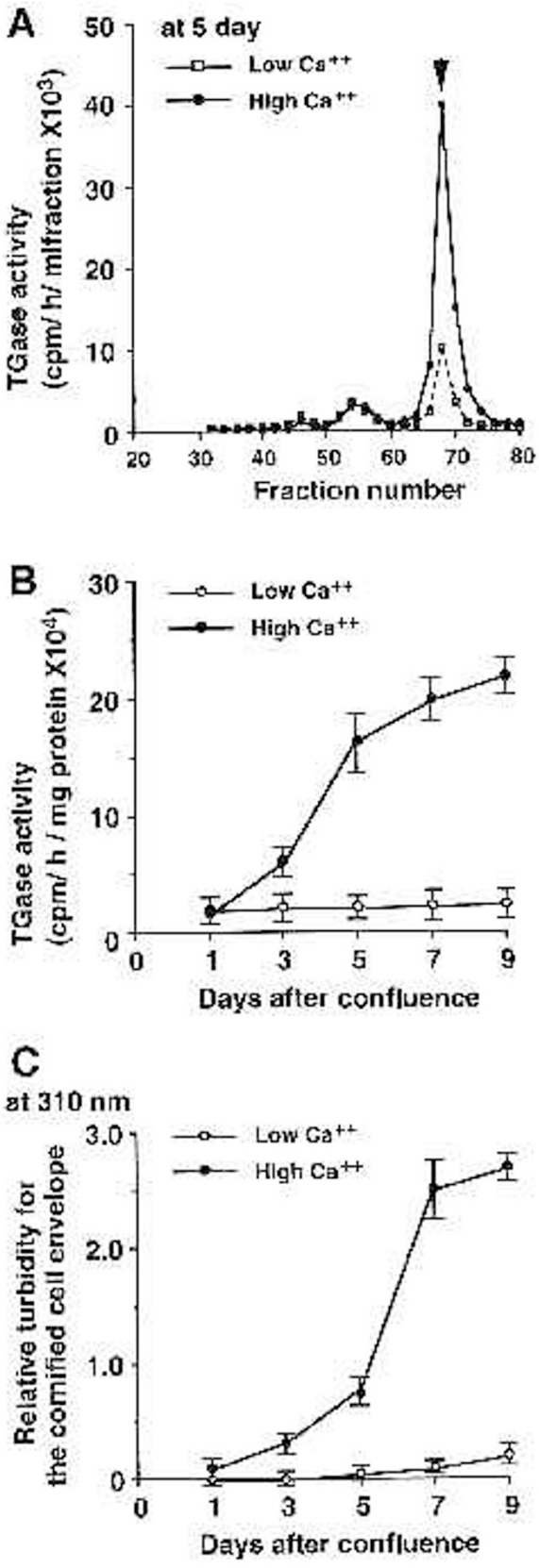

Figure 1. TGase 1 activation correlated with insoluble cross-linked CE formation during calcium induced differentiation of NHEK. NHEK cells were grown under low (0.05 mM) and high (1.2 mM) calcium conditions to confluence. **A)** The cells were harvested and seperated on the Mono-Q HPLC column using a NaCl gradient after 5 days (see 'Materials and Methods'). The arrow head shows highly active processed TGase 1 (Kim *et al*., 1995). **B)** Each value represents the highly active TGase peak from panel A at numbered days after induced differentiation under high calcium conditions. **C)** The CE was purifed and measured for relative turbidity using a spectorphotometer at 310 nm at numbered days after induced differentiation under high calcium conditions.

Protease inhibitors treatment of the NHEK cells

Normal human epidermal keratinocytes (NHEK, Clonetics, San Diego, CA, U.S.A.) were grown to confluency in a medium containing 0.05 mM $CaCl_2$ as recommended by the manufacturer. At 100% confluency, the calcium level was raised to 1.2 mM for the calcium induced differentiation and the cells were treated with various protease inhibitors in a concentration range of 0.5 μM to 0.5 mM including aprotinin, leupeptin (Acetyl-leu-leu-arg-al) and calpain I inhibitor (N-acetyl-leu-leu-norleucinal) (Boehringer-Mannheim Co, Indianapolis, IN, U.S.A.). Following 1, 3, 5, 7 and 9 days of treatment, the cells were harvested by scraping, sonicated, and incubated for 15 min at 4˚C in a buffer containing 0.1 M Tris-acetate, 0.1% Triton X-100 0.15 M NaCl, 1 mM and EDTA (TTBS) (4×10^8 cells/ml). The lysate was clarified by centrifugation at 10,000 *g* for 20 min at 4˚C to obtain the total extraction of the cells (Steinert *et al*., 1996). After centrifugation at 10,000 *g* for 10 min, the total fraction was subjected to chromatography on a 0.5×5 cm mono-Q HPLC column using 50 ml of a 0 to 0.5 M NaCl linear gradient (0.5 ml/min, 0.5 ml fractions) (Kim *et al*., 1990). Aliquots from every other fraction were used for TGase activity measurements. Three peaks of activity were identified (panel A of Figure 1), which were then separately pooled. The main peak of TGase activity in each treatment group was measured and then normalized by protein amount throughout the treatment days (panel B of Figure 1), which corresponded to the active processed TGase 1 (Kim *et al*., 1995). The immunoprecipitation was performed using polyclonal anti-TGase1, immobilized to protein G-agarose as described before (Kim *et al*., 1994). The immunoprecipitates were used for gel electrophoresis (panel D of Figure 2).

Preparation of CE from cultured human keratinocyte

We used a calcium-induced differentiation model of

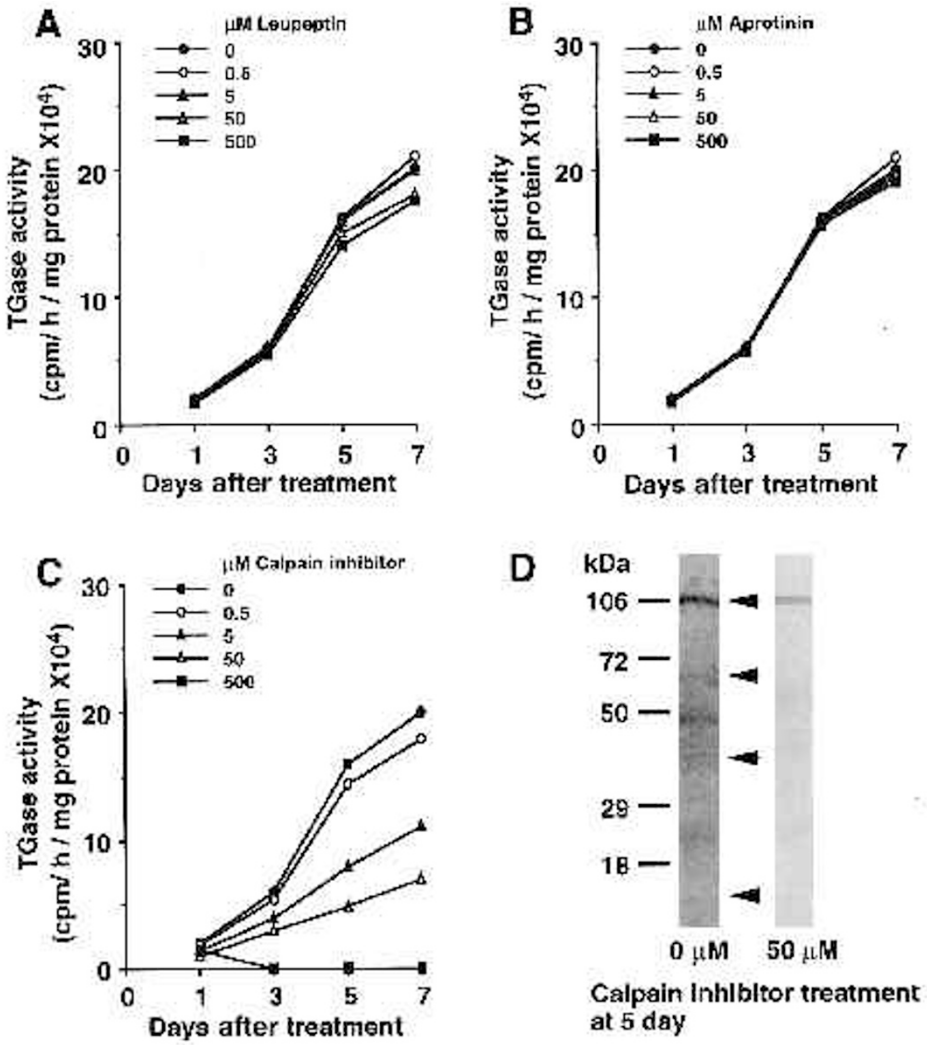

Figure 2. Dose-dependent inhibitory effects of TGase activity using various protease inhibitors on calcium induced differentiating NHEK cells. Leupeptin **(A)**, aprotinin **(B)**, and calpain I inhibitor **(C)** were treated in the culture media seperately in concentrantions ranging from 0.5 to 500 μM. **(D)** The coomassie staining of immunoprecipitation using anti-TGase 1 with or without treatment with calpain inhibitor (50 μM) was performed. Without treatment, multiple processed bands at 67, 33, 10 kDa including a 106 kDa intact band (arrow heads) were noticed as reported (Kim *et al*., 1995) while no processed form was observed during treatment.

keratinocytes as described, (Kim *et al*., 1995) was employed. The peptides inhibitors were added to the media at several different concentrations from 50 μM to 50 mM. At 1, 3, 5, 7 and 9 days of treatment, the cultured keratinocytes were harvested by scraper and the CE was purified by the established method (Schimidt *et al*., 1988). The purified CE was dissolved in 1 ml TTBS buffer/1.6×10^7 cells and sonicated twice for 30 sec at 70-90 watts. Turbidity of the insoluble CE at 310 nm was measured.

Results and Discussion

The proteolytic activation of increased TGase 1 expression is responsible for insoluble CE formation during calcium induced differentiation

NHEK cells can be differentiated under from low calcium (0.05 mM) to high calcium (1.2 mM) culture conditions. During this morphophysiological change, many structural proteins alter the expression. Keratin 5 and 14 expression in the proliferating NHEK cells switch to keratin 1 and 10 in differentiating cells (Fuchs and Green, 1980, 1981). Also terminal differentiation related proteins start being expressed to build a scaffold of barrier structures such as loricrin, filaggrin, small proline rich proteins, envoplakin, elafin, and involucrin (Steinert and Marekov, 1995). These structural proteins form envelopes in a highly ordered manner and finally are cross-linked by TGase (Steinert *et al*., 1998). Though there are three TGase isotypes in the human skin, over 90 % of TGase messages are TGase 1 in cultured NHEK cells under high calcium conditions (Steinert *et al*., 1996). We showed that TGase activity is closely related with CE formation. The profile of TGase activity in panel A of Figure 1 showed that the most TGase activity after calcium induced differentiation corresponds to processed TGase 1 (Kim *et al*., 1995). This activity peak was increased up to 10 folds after 7 day culture in high calcium compared to low calcium conditions (panel B of Figure 1). However, between 3 and 5 days, TGase 1 activity exponentially increased. Interestingly the insoluble CE exponentially increased up to 27 folds between 5 and 7 days under high calcium conditions (panel C of Figure 1). This suggests that TGase 1 activity might increase 24 to 48 h prior to the increase of CE.

Calpain I inhibitor reduced TGase 1 activity by inhibiting TGase 1 processing in differentiating NHEK cells

Our previous study showed that proteolytic activation was required to obtain high functional activity of TGase 1 *in vitro* or in NHEK cells (Kim *et al*., 1995; Kim *et al*., 1996). Hence, we employed the several protease inhibitor treatments on the NHEK cells during the calcium induced differentiation. Leupeptin was selected for inhibiting serine thiol proteases such as trypsin, plasmin, proteinase K, kallikrein, papain, thrombin, and cathepsin A and B. Apro-tinin, a known as serine protease inhibitor, was selected to inhibit plasmin, kallikrein, trypsin, and chymotrypsin. N-Acetyl-Leu-Leu-norleucinal (calpain I inhibitor) was selected to inhibit calpain I and II, since there was a report of calpain I and II expression in skin (Miyachi *et al*., 1986). Though calpain I inhibitor was developed for strong competitive inhibitor of the calcium dependent neutral cysteine protease calpain I and II, it can also be a strong inhibitor of papain and cathepsin B and L. Panel A of Figure 2 shows that 0.5 mM leupeptin treatment reduced by only 10% TGase 1

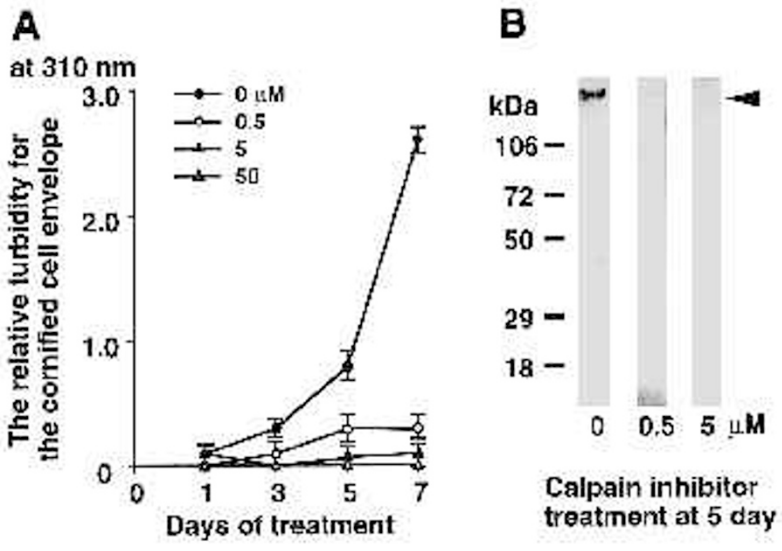

Figure 3. The inhibitory effect of CE formation in NHEK cells by treatment with calpain I inhibitor. **(A)** The inhibitor was in the concentration range of 0.5 to 50 μM. CE was purified and measured at 310 nm after inhibitor treatment under high calcium conditions (details in 'Materials and Methods'). **(B)** Coomassie staining of purified CE was shown mostly in the stacking gel due to its high molecular weight (arrow head) after 10% tris-glycine SDS-polyacrylamide gel elctrophoresis.

activity while panel C shows dramatic decrease of TGase 1 activity by almost 100% on day 3 of treatment with calpain I inhibitor. We selected samples at day 5 from control and 50 μM calpain I inhibitor treated cells for immunoprecipitations using anti-TGase 1 (panel D of Figure 2). This gave processed TGase 1 bands at 67, 33, 10 kDa including intact 106 kDa (arrow heads) from the non-treated sample while a calpain I inhibitor treated sample gave only the intact form of TGase 1. Even though leupeptin and aprotinin did not inhibit TGase 1 activity, there is a possibility that these inhibitors were more unstable than calpain inhibitors.

Calpain I inhibitor reduced insoluble CE formation

Insoluble CE was purified from the same experimental sets of NHEK cells as panel C of Figure 2. Surprisingly Calpain I inhibitor was very effective in preventing the formation of insoluble CE (panel A of Figure 3). At day 7, 0.5 μM calpain I inhibitor inhibited about 90 % of CE formation. This strong efficacy might be due to the small molecular weight and non-charged molecular structure of the calpain I inhibitor. The purified insoluble CE from control, 0.5 and 5 μM calpain I inhibitor treated group was run on a 10 % tricine-SDS acrylamide gel (panel B of Figure 3). The CE could not penetrate into the stacking gel due to highly cross-linked polymers (arrow head). This was reduced to the level of production under low calcium conditions by 5 mM calpain inhibitor treatment. This result suggests that the differentiation rate might be closely related to functional calpain content in keratino-cytes (Miyachi *et al*., 1986). In the inhibitor treatment of cultured cells, we observed no side effects such as cell detachment or apoptosis (data not shown). Therefore, calpain inhibitors might be useful in reducing insoluble corneum in hyperkeratotic skin lesions. However, there are obvious limitations to the use of calpain inhibitor I both as a pharmacological tool and in medicine, since its selectivity is lower than *in vivo* inhibitor calpastatin (Wang, 1990). Further study will determine the mechanism of how and when calpain recognizes TGase 1 as an endogenous substrate.

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