Title: Green Tea Polyphenols Induce Differentiation and Proliferation in Epidermal Keratinocytes

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Running Title: **Tea Polyphenol-Induced Differentiation & Proliferation**

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Number of text pages: 25

Number of tables: 0

Number of figures: 4

Number of references: 30

Number of words in the *Abstract:* 151

Number of words in the *Introduction*: 652

Number of words in the *Discussion*: 1047

List of abbreviations: GTPPs: green tea polyphenols; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; EGCG: -(-) epigallocatechin-3-gallate.

Recommended section: Cellular and Molecular

JPET Fast Forward. Published on March 27, 2003 as DOI: 10.1124/jpet.103.049734
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The most abundant green tea polyphenol, epigallocatechin-3-gallate (EGCG), was found to induce differential effects between tumor cells and normal cells. Nevertheless, how normal epithelial cells respond to the polyphenol at concentrations for which tumor cells undergo apoptosis is undefined. The current study tested exponentially growing and aged primary human epidermal keratinocytes in response to EGCG or a mixture of the four major green tea polyphenols. EGCG elicited cell differentiation with associated induction of p57/KIP2 within 24 hours in growing keratinocytes, measured by the expression of keratin 1, filaggrin and transglutaminase activity. Aged keratinocytes, which exhibited low basal cellular activities after culturing in growth medium for up to 25 days, renewed DNA synthesis and activated succinate dehydrogenase up to 37-fold upon exposure to either EGCG or the polyphenols. These results suggest that tea polyphenols may be used for treatment of wounds or certain skin conditions characterized by altered cellular activities or metabolism.

Green tea polyphenols (referred to as GTPPs, to include the four major polyphenols [-]-Epicatechin [EC], [-]-Epigallocatechin [EGC], [-]-Epcatechin-3-gallate [ECG] and [-]-Epigallocatechin-3-gallate [EGCG]) have been identified to possess chemopreventive and apoptotic activity against certain cancers, while normal epidermal keratinocytes follow a survival pathway that has not been fully elucidated (Ahmad and Mukhtar H. 2001; Chen et al., 1998; Hsu et al., 2001; Hsu et al., 2003; Hsu et al., 2002a; Hsu et al., 2002b; Kuroda and Hara, 1999; Mukhtar and Ahmad, 1999; Mukhtar and Ahmad, 2000; Pasta et al., 1998). The most abundant green tea polyphenol, epigallocatechin-3-gallate (EGCG), was reported to induce differentiation and decreased cell proliferation in epidermal keratinocytes [Balasubramanian et al., 2002], rather than apoptosis as it does in tumor cells [Hsu et al., 2001; Hsu et al., 2003; Hsu et al., 2002a, Paschka et al., 1998].

We previously reported that both GTPPs and EGCG were able to induce transient expression of p57/KIP2, a differentiation/cell cycle regulator, which was associated with cell survival during GTPP-exposure [Hsu et al., 2001]. It was proposed that p57 induction may stimulate cell differentiation as part of a survival pathway [Hsu et al., 2001; Hsu et al., 2002a; Hsu et al., 2002b]. While this survival pathway is currently under investigation, the impact of GTPPs on epidermal keratinocytes located in various layers of the skin was deemed essential to be addressed, given the fact that GTPPs are able to penetrate the epidermis, but not the dermis, of human skin [Dvorakova et al., 1999].

Keratinocytes within the epidermis exist in various stages of differentiation corresponding to different epidermal layers [Bollag and Bollag, 2001, Bikle et al., 2001]. For example, the basal keratinocytes and/or stem cells at the dermal-epidermal junction continuously proliferate to regenerate and restore cells lost to the environment. As the daughter cells migrate up through the epidermal layers, they first undergo growth arrest followed by expression of keratins 1 and 10 in the spinous layer. In the next layer, the granular layer, late markers of keratinocyte differentiation, including filaggrin and other structural proteins, are expressed. In addition, the activity of transglutaminase, the enzyme that cross links the structural proteins into the cornified envelope, is increased. Finally, the keratinocytes undergo an epidermal-specific programmed cell death to form the cornified layer, which serves as a barrier to mechanical injury, microbial invasion and water loss. The entire epidermis turns over in one to two months, although the transit time of keratinocytes may be lengthened or shortened in various disease states. We considered it pertinent to investigate whether GTPPs induce differential effects among keratinocytes at different stages of differentiation and/or age, knowing that if so, such effects could be significant for assessing the potential impact of these compounds upon topical application. Thus, agents that accelerate growth and/or differentiation of epidermal keratinocytes may shorten the healing time of certain wounds and serve as treatments for certain epidermal-skin diseases.

We hypothesized that green tea polyphenols, either in a mixture or in the form of purified EGCG, are able to increase cellular activities, including new DNA synthesis, in aged keratinocytes, or promote differentiation of exponentially growing keratinocytes located in the basal layer of epidermis. Therefore, the current study examined pooled normal human primary

epidermal keratinocytes treated with EGCG or GTPPs after various times of culture. Results from this study demonstrated that: 1) by promoting biological energy production and new DNA synthesis, both EGCG and GTPPs "re-energized" the aged keratinocytes; thus, these compounds can presumably stimulate the regeneration of keratinocytes in aging skin; 2) by induction of p57, keratin 1 and filaggrin expression, and activation of transglutaminase, EGCG also stimulated the differentiation of the keratinocytes found in the basal layer of the epidermis. The combination of these two effects may help to accelerate wound healing and regeneration of new skin tissue, and subsequently prevent scar tissue formation. In addition, certain epithelial conditions may be amenable to treatment by topical applications of green tea polyphenols.

MATERIAL AND METHODS

Chemicals and antibodies

EGCG was purchased from Sigma (St. Louis, MO). A mixture of four major green tea polyphenols (GTPPs) was purchased from LKT Lab, Inc (Minneapolis, MN). GTPPs and EGCG were dissolved in keratinocyte growth medium-2 (KGM-2, BioWhittaker) and filter-sterilized immediately prior to use. The rabbit anti-human p57 antibody C-19 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the rabbit anti-filaggrin and anti-keratin-1 antibodies were from Covance (Berkeley, CA).

Culturing normal human epithelial cells

The pooled normal human primary epidermal keratinocytes were purchased from BioWhittaker (East Rutherford, NJ) and sub-cultured in the specific growth media provided by the manufacturer (KGM-2). Subculture of the epithelial cells was performed by detaching the cells in 0.025% trypsin and transferring into new tissue culture flasks, at the recommended density of 3500 cells/cm^2. Exponentially growing keratinocytes were treated and harvested in their early passages (2-3 passages). Aged keratinocytes were allowed to grow in 96-well tissue culture plates for 15, 20 and 25 days prior to treatment by EGCG or GTPPs, followed by various assays.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

In a 96-well plate, cells were seeded at $1.5X10^4$ cells per well. After 24 h treatment, culture medium was removed and replaced with 100 µl of 2% MTT in a solution of 0.05 M Tris, 0.5 mM $MgCl_2$, 2.5 mM $CoCl_2$, and 0.25 M disodium succinate (Sigma, St. Louis, MO) and the

plate was incubated at 37^{o} C for 30 minutes. The MTT solution contains only disodium succinate as a substrate; therefore, this method directly detects the activity of mitochondrial succinate dehydrogenase (SDH). Cells were fixed *in situ* by the addition of 100 µl of 4% formalin in 0.2 M Tris (pH 7.7), and after a 5 min incubation at room temperature liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 µl water and cells were solubilized by the addition of 100 µl of 6.35 % 0.1 N NaOH in DMSO. The colored formazan product was measured by a Thermo MAX micro plate reader (Molecular Devices Corp. Sunnyvale, CA) at a wavelength of 562 nm. Experiments were repeated three times with triplicate samples for each experiment.

Analysis of DNA synthesis using the BrdU incorporation method

The BrdU cell proliferation kit was purchased from Oncogene Research Products (Boston, MA). Cells were cultured in 96-well plates at the density of 10^4 cells/well. After EGCG and GTPPs treatments, cells were labeled with BrdU for 12 hours and levels of BrdU incorporation determined according to the manufacturer's instructions using a Thermo MAX micro-plate reader at a wavelength of 450 nm and subtracting absorbance measured at 540 nm. Experiments were repeated three times in triplicate for each experiment.

Immunocytochemistry

Normal human keratinocytes were seeded in 8-well chamber slides (Nagle Nunc International, Naperville, IL) 12 h prior to EGCG treatment. At the end of a 24 h treatment, the slides were washed with PBS and fixed in a cold 4% paraformaldehyde solution for 10 min. Then 3% hydrogen peroxide solution and normal goat serum were applied to block endogenous peroxidase

activity and non-specific binding. The primary antibodies, rabbit-anti-human p57 polyclonal antibody C-19, rabbit anti-human keratin 1 and filaggrin antibodies were applied for 1 h at 37^{o} C at the dilutions recommended by the manufacturers. The Streptavidin detection technique (Biogenex, USA) was used with 3-amino-9-ethylcarbazole as chromogen. Negative control sections consisted of cells treated with 1% diluted normal goat serum instead of primary antibody. Mayer's hematoxylin was used as a counter-stain.

Transglutaminase activity assay

Normal human epidermal keratinocytes in early passages were allowed to grow in 6-well tissue culture plates prior to EGCG exposure. The cells were scraped in homogenization buffer (0.1 M Tris/acetate, pH 8.5, containing 0.2 mM EDTA, 20 µM AEBSF, 2 µg/mL aprotinin, 2 µM leupeptin and 1 µM pepstatin A), collected by centrifugation and subjected to one freeze-thaw cycle prior to lysis by sonication. Unlysed cells were pelleted by centrifugation and aliquots of the supernatant collected for the determination of transglutaminase activity and protein concentration. Protein quantities were determined using the BioRad Protein Assay with bovine serum albumin as standard. Transglutaminase activity was measured as the incorporation of [^{3}H] putrescine into dimethylated casein, as described previously [Jung et al., 1998].

Caspase 3 activity assay

The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. Cells (10^{5} per well) were plated in triplicate in a 24-well tissue culture plate. After 24 hr treatments with

EGCG or GTPPs, the cells in each well were washed with 1 ml PBS and incubated with 100µl lysis buffer on ice for 10 min. To each well, 100 µl of 2X reaction buffer was added with 10 mM DTT. Finally, 5 µl of DEVD-AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 hour at 37º C, and caspase 3 activity in each well was measured using a fluorescence micro-plate reader at a wavelength of 405 nm for excitation and 505 nm for emission.

RESULTS

Unlike a variety of tumor cell types tested, normal human epidermal keratinocytes are able to survive when exposed to EGCG or GTPPs [Chen et al., 1998; Hsu et al., 2001; Jin et al., 2001; Mukhtar and Ahmad, 1999; Nie et al., 2002; Suganuma et al., 1999]. Therefore, we examined the effect of EGCG and GTPPs on markers of cell status, such as apoptosis, proliferation, differentiation and energy production. In initial experiments, exponentially growing keratinocytes were exposed to these agents. As shown in Figure 1 there was no effect of EGCG or GTPPs on caspase 3 activity, a measure of apoptosis. There was also little or no effect of these compounds on BrdU incorporation into DNA (Figure 1), a measure of proliferative status, or on energy production, as measured by changes in the mitochondrial enzyme succinate dehydrogenase (SDH) activity monitored by the MTT assay (Figure 1).

Treatment with EGCG was observed to induce changes in cell morphology as shown in Figure 2. Thus, EGCG increased the number of enlarged, flattened, squame-like cells observed in these cultures (Figure 3B). This morphology is typical of differentiated keratinocytes, suggesting an ability of EGCG to trigger cell differentiation. Further confirmation of this capacity of EGCG to induce keratinocyte differentiation is provided by the results shown in Figure 2C. These data demonstrate that in exponentially growing keratinocytes EGCG was also able to increase, by up to 500%, transglutaminase activity, a characteristic of late-stage keratinocyte differentiation.

Our previous results suggested that in keratinocytes EGCG- and GTPPs-mediated induction of the expression of a cell cycle inhibitor p57 allowed survival of these cells upon exposure to the polyphenols (Hsu et al, 2001; Hsu et al, 2002b). Therefore, we examined the effect of EGCG

and GTPPs on p57 expression in exponentially growing keratinocytes. Figure 3B shows that EGCG induced p57 expression in these cells. In addition, EGCG enhanced the expression of an early keratinocyte differentiation marker, keratin 1 (Figure 3D), as well as a late marker, filaggrin (Figure 3F), suggesting that this agent had the capacity to induce multiple stages of keratinocyte differentiation.

Since responses of aged keratinocytes may differ from those of exponentially growing keratinocytes, we also investigated the effect of EGCG and GTPPs on keratinocytes that were no longer in an exponential growth phase. After 15, 20, or 25 days in culture, the pooled primary human epidermal keratinocytes gradually lost their ability to either generate ATP or divide. At these time points, EGCG or GTPPs was able to activate SDH, as measured by the MTT assay (Figure 4A, C and E), up to 37-fold (25 days, Figure 4E). The activation of this component of the tricarboxylic acid (TCA) cycle may provide biological energy and substrates for other responses such as new DNA synthesis. When aged human keratinocytes lost the ability to synthesize new DNA, especially after 20 + days in KGM-2, both EGCG and GTPPs were able to stimulate new DNA synthesis, as measured by BrdU incorporation into DNA (Figure 4B, D and F), up to approximately 3-fold (25 days, Figure 4F). To our knowledge, this represents the first observation that green tea components stimulate energy generation in aged epidermal keratinocytes. On the other hand, it was noted that for the aged keratinocytes at the 15-day and 20-day time points, lower concentrations of EGCG (15-50 μM) had a slight negative impact on BrdU incorporation (Figure 4B and D). Nevertheless, EGCG concentrations higher than 100 μM consistently induced both SDH activity and BrdU incorporation (Figure 4).

DISCUSSION

Previous reports describing growth arrest and differentiation of exponentially growing keratinocytes indicated that EGCG enhanced the expression of involucrin and increased the conversion of undifferentiated keratinocytes into corneocytes with concomitant decreased cell proliferation [Balasubramanian et al., 2002]. The current study further confirmed that undifferentiated keratinocytes were able to commit to differentiation upon EGCG treatment within a short period of time, accompanied by an elevation in the activity of transglutaminase, the enzyme that cross-links involucrin and other substrates to form the cornified envelope [Bikle et al., 2001]. When exponentially growing pooled normal human primary epidermal keratinocytes were incubated with 50-100 μM EGCG, these cells underwent differentiation in 24 h, as measured by immunocytochemistry using antibodies against keratin 1 (an early differentiation marker), filaggrin (a late differentiation marker) (Figure 3), and transglutaminase activity assay (a late differentiation marker) (Figure 2). EGCG concentrations of 50 -100 μM were adequate to induce cell differentiation and were accompanied by a marked elevation in p57 (a differentiation/growth arrest inducer also known as KIP2), indicating p57 may not only be responsible for cell survival but also for cell differentiation [Hsu et al., 2001; Hsu et al., 2002a; Hsu et al., 2002b]. Thus, the ability of keratinocytes to survive treatment with EGCG and GTPPs might be related to this induction of p57. On the other hand, this survival capacity may involve regulation of pro-survival factors, cell cycle factors and/or cell differentiation factors at the transcriptional and/or translational level [Ahmad et al., 1997; Ahmad et al., 2000; Balasubramanian et al., 2002; Chen et al., 2000; Chen et al., 1999; Hsu et al., 2001; Hsu et al., 2002a; Hsu et al, 2002b]. The mechanism of the survival pathway is currently under active investigation.

The EGCG concentrations used are within the physiological range in humans, given the fact that daily topical application of 30 mg/ml EGCG (655 times higher than 100 μM) for 30 days failed to induce dermal toxicity [Stratton et al., 2000]. In addition, the viability of the keratinocytes was confirmed by BrdU incorporation and SDH activity upon EGCG or GTPPs exposure, and their apoptotic status investigated by a caspase 3 activity assay; and there was no major alteration in these measurements (Figure 1). In contrast, this same concentration range has been applied to tumor cell cultures and induced apoptotic effects [Chen et al, 2000; Jin et al., 2001; Nakagawa et al., 2002; Nie et al., 2002; Suganuma et al., 1999; Yakoyama et al., 2001]. This evidence suggests that the effect of EGCG on exponentially growing keratinocytes is a selective induction of differentiation, in contrast to the apoptotic cell death initiated in tumor cells.

It has been suggested that H_2O_2 produced by EGCG in cell culture is an important factor for tumor cell death (Sakagami et al., 2001). At concentrations above 50 μM, EGCG is able to produce considerable amounts of H_2O_2 and form oxidized products in cell culture that may interfere with MTT assays (Hong et al., 2002). We found that incubation of keratinocytes with H_2O_2 concentrations reported to be induced by EGCG for time periods that paralleled those of Hong's study did not influence the MTT assay under our experimental conditions. Likewise, MTT assay results were not significantly higher at the 4 h EGCG-treatment time point (data not shown), when the oxidized products of EGCG peak (Hong et al., 2002). In addition, MTT assays were carried out with or without MTT/sodium succinate, the results indicated that EGCG and/or its oxidized forms do not play a significant role to 562 nm absorption in our system (data not shown).

Results in Figures 2 and 3 indicated that EGCG induce differentiation in exponentially growing keratinocytes whereas those in Figure 4 suggested that in aged keratinocytes the effect of EGCG and GTPPs was to stimulate proliferation and accelerate TCA cycle that supplies reducing equivalents for biological energy production. Therefore, the age of the keratinocytes and the concentration of EGCG or GTPPs used are two key factors in terms of the effects of these agents on differentiation, energy generation and DNA replication. Of interest is the relationship of aged cultures of keratinocytes to their differentiation status. Since human keratinocytes are prone to undergo growth arrest and to express differentiation markers upon attaining confluence [Lee et al., 1998], we predict that the response of keratinocytes in upper epidermal layers will mirror that of the aged keratinocytes. Thus, we propose that EGCG and the GTPPs will stimulate reentry into the cell cycle in the early differentiated (spinous) stratum of the skin. A study by Fu et al. [2000] also suggests a protective effect of GTPPs on rat keratinocytes, such that these authors observed a decrease in an apoptotic marker (lactate dehydrogenase release) as well as an increase in keratinocyte proliferation as measured by cell counting, flow cytometry and proliferative index. Additional protective effects of GTPPs are also indicated by their ability to act as antioxidants and decrease lipid peroxidation products and increase glutathione peroxidase levels [Fu et al., 2000]; and their ability to protect the epidermis from carcinogen-induced tumorigenesis or chemically induced inflammation (Huang et al., 1992).

Thus, we report here for the first time, that at certain concentrations, EGCG or a mixture of the major green tea polyphenols stimulated aged keratinocytes to generate biological energy and to synthesize DNA, possibly for renewed cell division. For keratinocytes in an exponential growth phase, EGCG potently stimulated these cells to commit to differentiation with minimal impact on DNA synthesis or energy levels during a 24-h period. We speculate that by simulating

differentiation of keratinocytes in the basal layer of the epidermis and energizing and stimulating cell division/DNA synthesis in aged suprabasal keratinocytes, these compounds could potentially reduce the time of healing and prevent the formation of scar tissue, which occupies the space not repopulated by keratinocytes. Therefore, green tea constituents may be useful topically for promoting skin regeneration, wound healing or treatment of certain epithelial conditions such as aphthous ulcers, psoriasis, rosacea and actinic keratosis. In addition, the differentiation-inducing potential of green tea components might be beneficial to patients who have conditions characterized by abnormally accelerated skin cell growth and lack of differentiation. However, future studies are needed to determine the optimal time of treatment, concentrations and combinations of green tea polyphenols to be used topically for therapeutic intervention in various epithelial conditions.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Petra Lockwood for the technical assistance and Mr. Philip Jones for the art work.

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FOOTNOTES

This study was supported by a grant from the Dental Research Foundation, and funding through the Department of Oral Biology and Maxillofacial Pathology, School of Dentistry, Medical College of Georgia, to SH.

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JPET Fast Forward. Published on March 27, 2003 as DOI: 10.1124/jpet.103.049734
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LEGENDS OF FIGURES

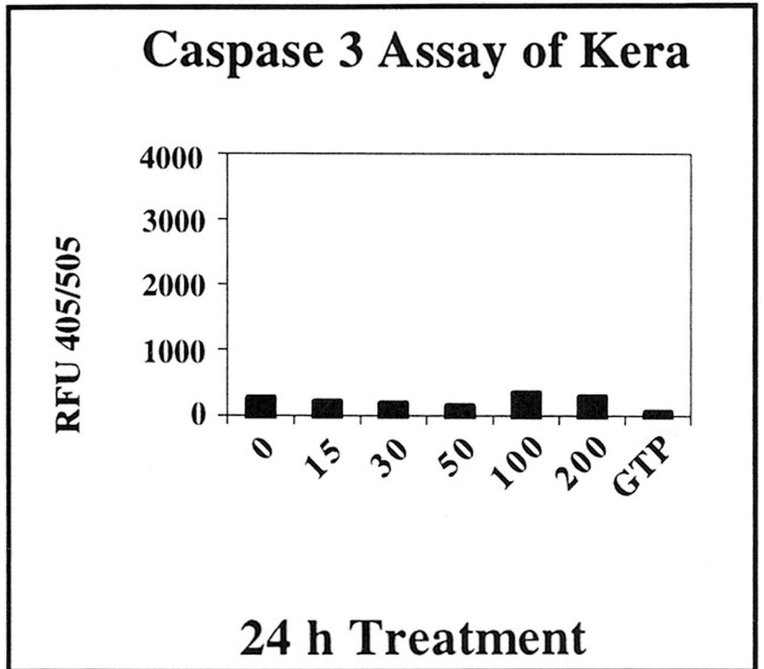
Figure 1. EGCG and GTPPs Do Not Alter Apoptosis or Mitochondrial Energy Production and Exert Minimal Effects on DNA Synthesis in Exponentially Growing Keratinocytes. Exponentially growing normal human primary epidermal keratinocytes were evaluated for caspase 3 activity, DNA synthesis and SDH activity following treatment with increasing concentrations of EGCG as indicated or 0.2 mg/ml GTP. The results showed that the caspase 3 activities and SDH activities were not significantly affected, while the BrdU assay demonstrated a slight increase in BrdU incorporation. Data represent the average with SD of triplicate samples. All experiments were performed three times with similar results.

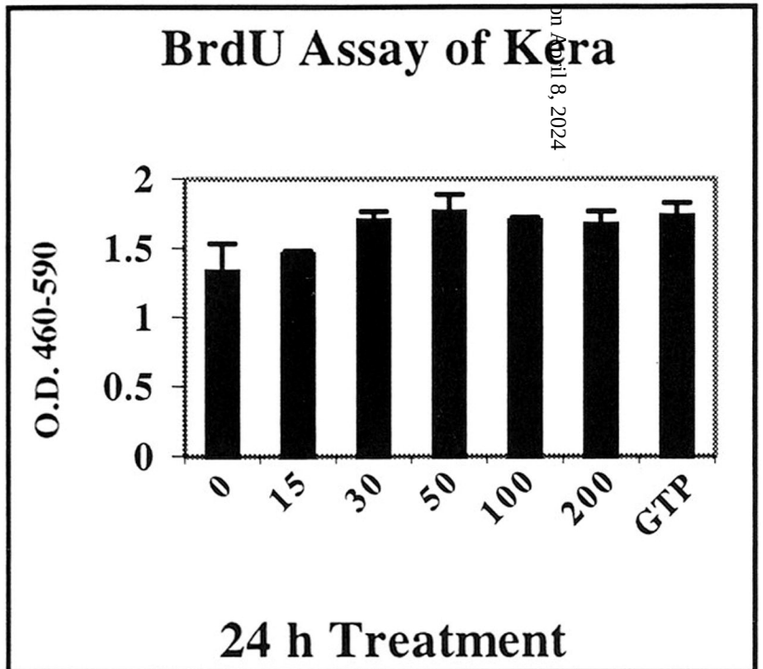
Figure 2. EGCG and GTPPs Stimulate Transglutaminase Activity in Exponentially Growing Keratinocytes. Panel **(A)** shows pre-confluent, exponentially growing normal human primary epidermal keratinocytes without treatment, whereas panel **(B)** illustrates pre-confluent normal human primary epidermal keratinocytes treated with 50 μM EGCG for 24 h. Morphological changes are evident, which suggests the cells have committed to differentiation. **(C)** Transglutaminase activity (a late differentiation marker) is compared between control and EGCG-treated cells. Cells treated with 50 μM or 100 μM EGCG have significantly higher activities. Data represent the average and standard deviation of triplicate samples with *$p<0.01$ versus the control value. Experiments were repeated three times with similar results.

Figure 3. EGCG and GTPPs Increase Levels of Differentiation Markers in Exponentially Growing Keratinocytes. Panels **(A)**, **(C)** and **(E)** show untreated cells. **(B)** Cells were treated

with 100 μM EGCG for 24 h and stained with antibody specific for human p57. **(D)** Cells were treated with 100 μM EGCG and stained with antibody specific for human keratin 1. **(F)** Cells were treated with 100 μM EGCG and stained with antibody specific for human filaggrin. In all panels EGCG was shown to induce changes in cell morphology, increasing the number of enlarged, flattened, squame-like keratinocytes observed in the cultures. These cell characteristics are typical of differentiated keratinocytes, as indicated also by the enhanced expression of differentiation markers in these squamous cells. Original magnification: 400X. Experiments were repeated twice with similar results.

Figure 4. EGCG and GTPPs Stimulate Mitochondrial Energy Production and DNA Synthesis in Aged Keratinocytes. Panels **(A)**, **(C)** and **(E)** show the results of MTT assays in normal human primary epidermal keratinocytes cultured for 15 days, 20 days or 25 days in KGM-2 medium, respectively, and treated with increasing concentrations of EGCG as indicated, or 0.2 mg/ml GTPPs for 24 h. Data represent the average and standard deviation of triplicate samples with *$p<0.05$ and **$p<0.001$ versus the control value. Experiments were repeated five times with consistent results. Panels **(B)**, **(D)** and **(F)** illustrate BrdU assay results of normal human primary epidermal keratinocytes cultured for 15 days, 20 days and 25 days in KGM-2 medium, respectively, and treated with increasing concentrations of EGCG as indicated or 0.2 mg/ml GTP for 24 h. Data represent the average and standard deviation of triplicate samples with *$p<0.01$ and **$p<0.001$ versus the control value. Experiments were repeated three times with consistent results, and the experiments were performed in parallel.





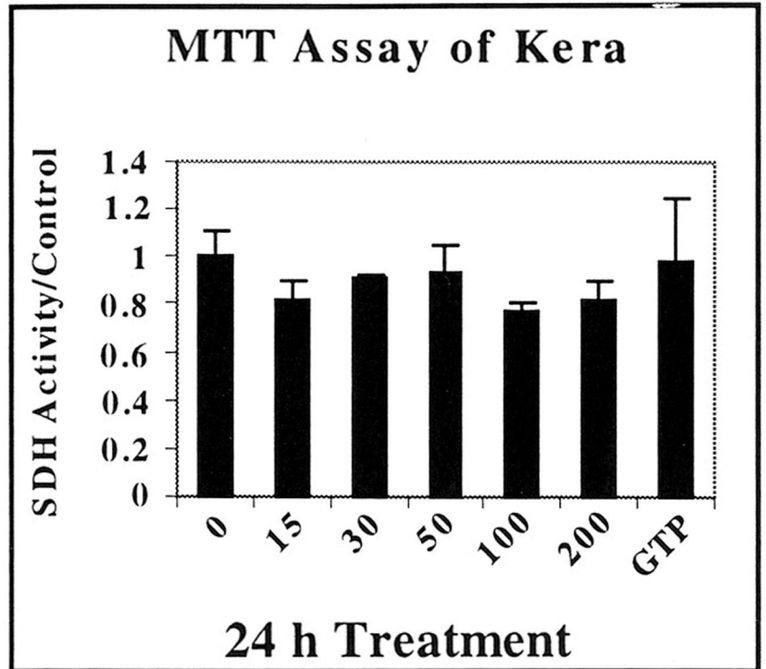


Fig. 1

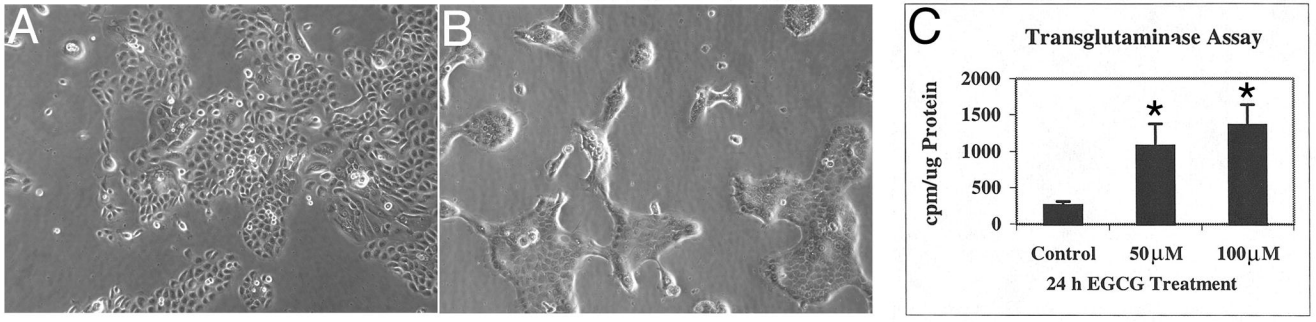

A
B
C
Transglutaminase Assay
cpm/ug Protein
2000
1500
1000
500
0
*
*
Control
50μM
100μM
24 h EGCG Treatment

Fig. 2

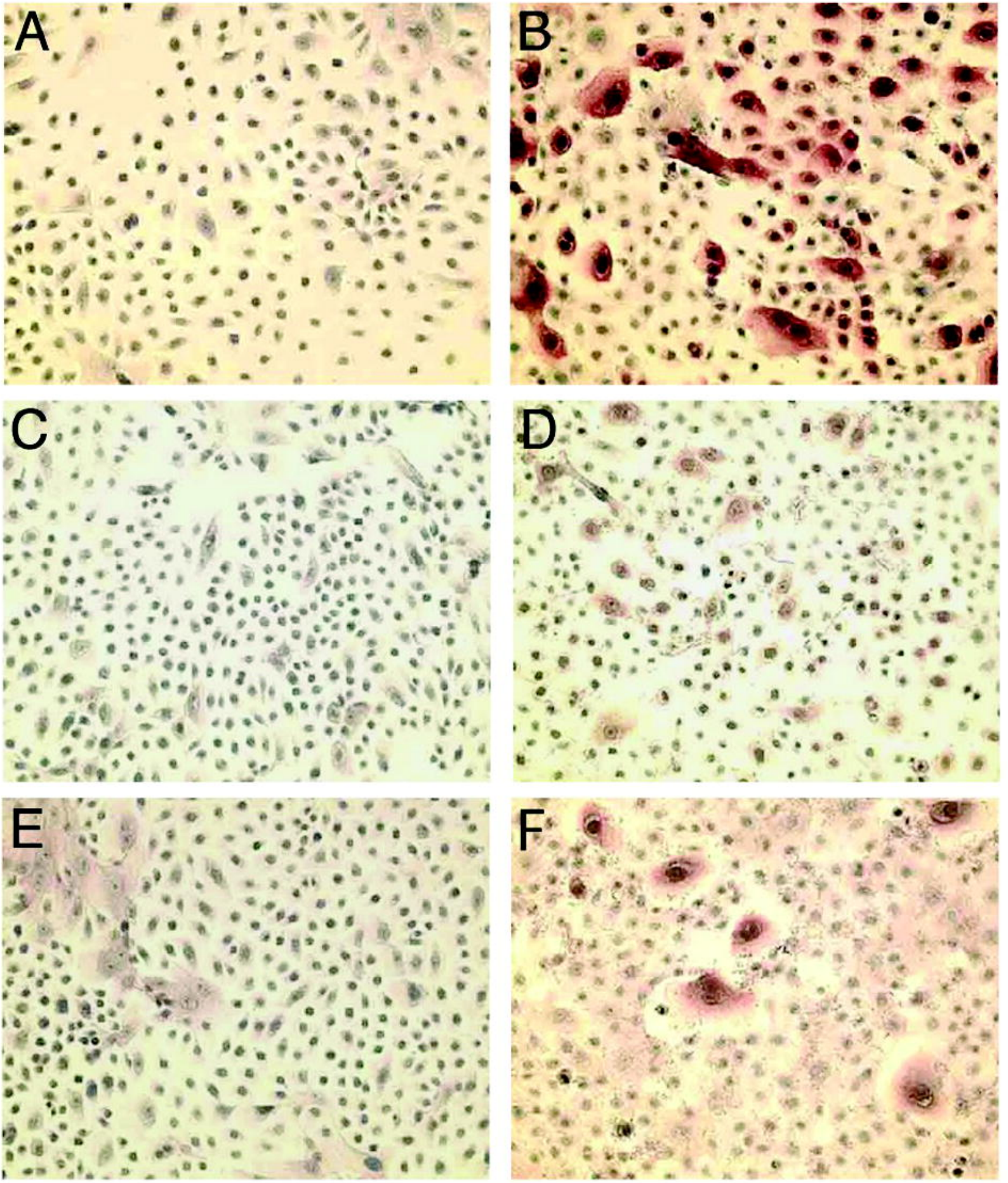
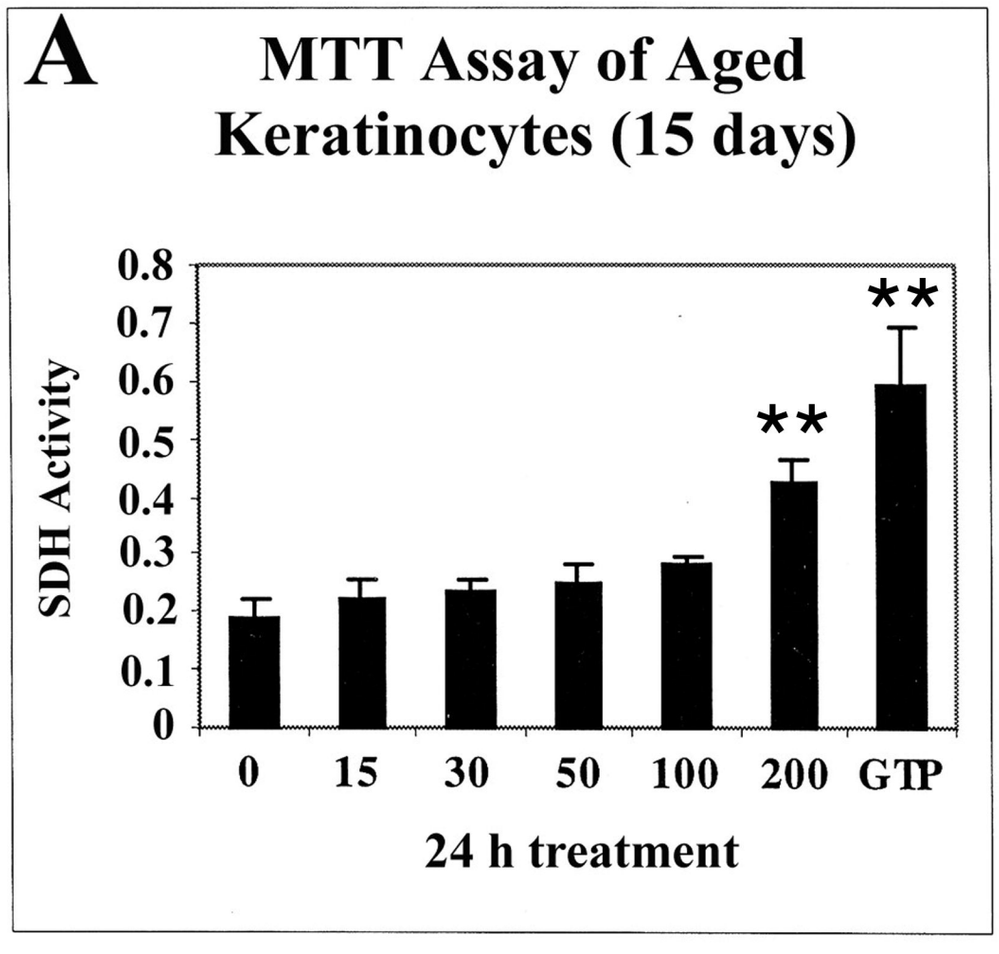
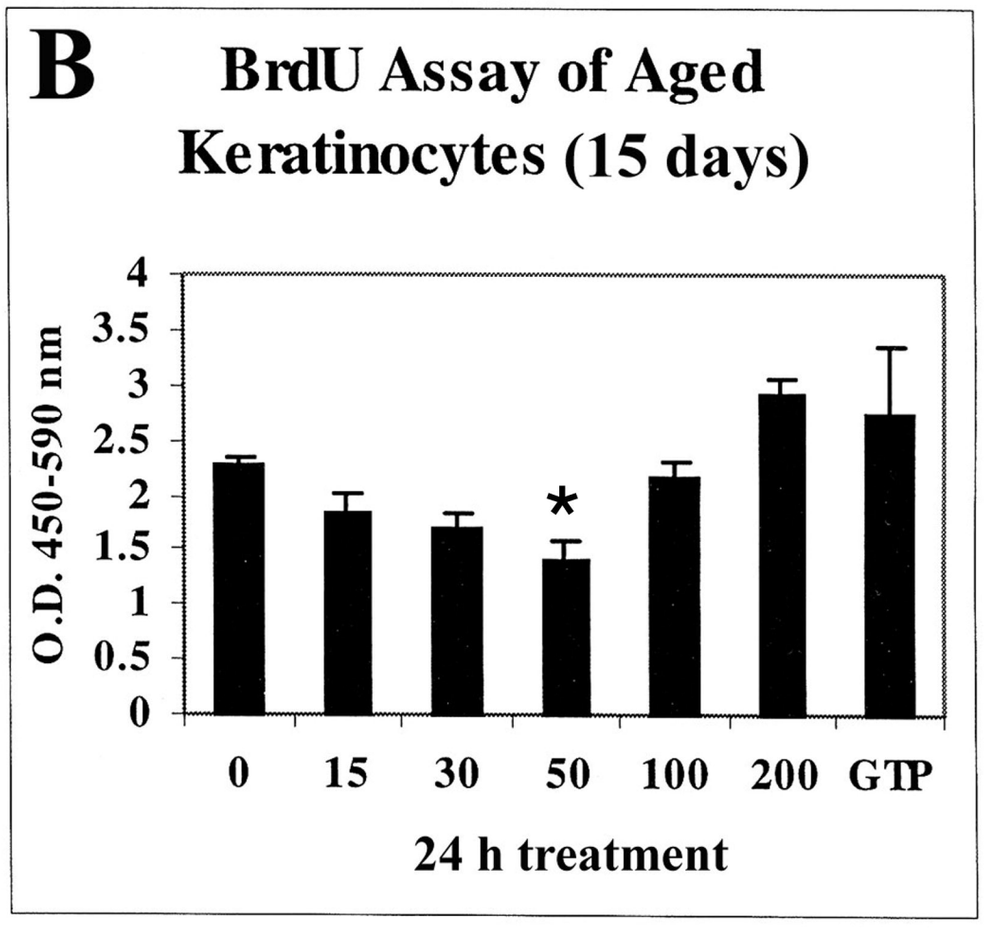
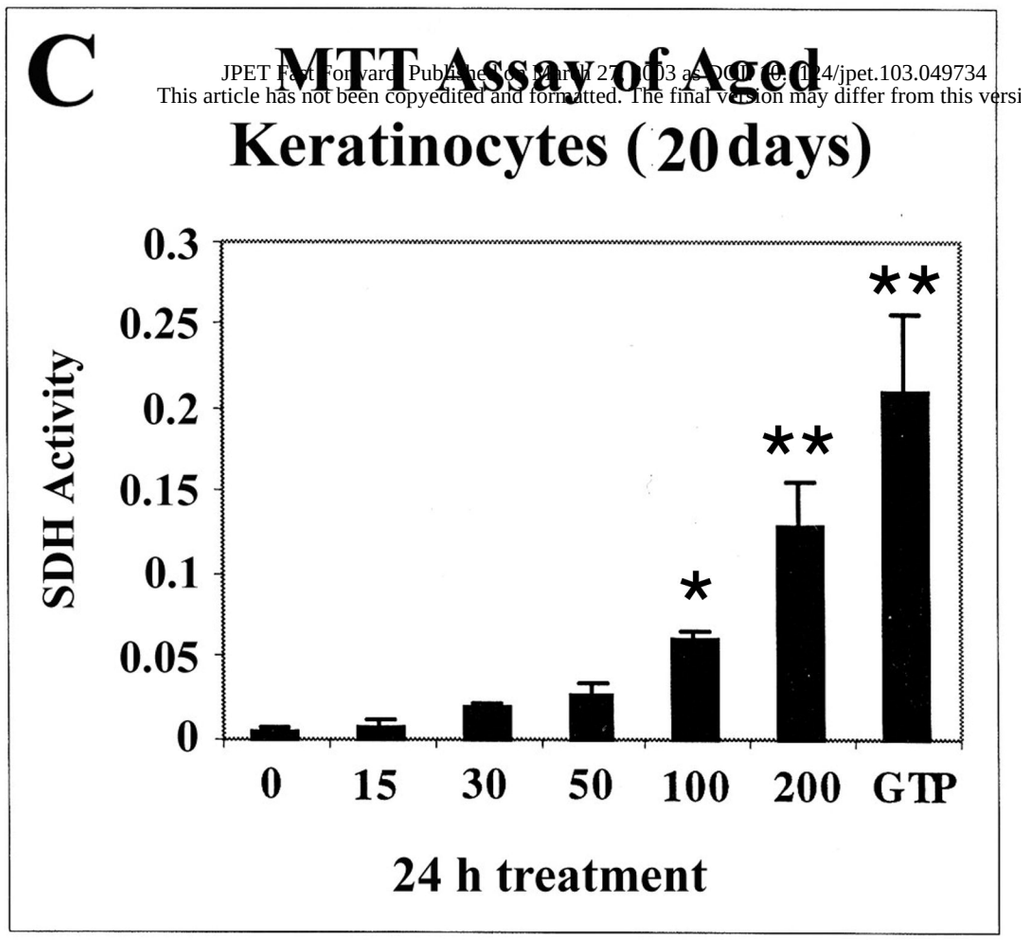
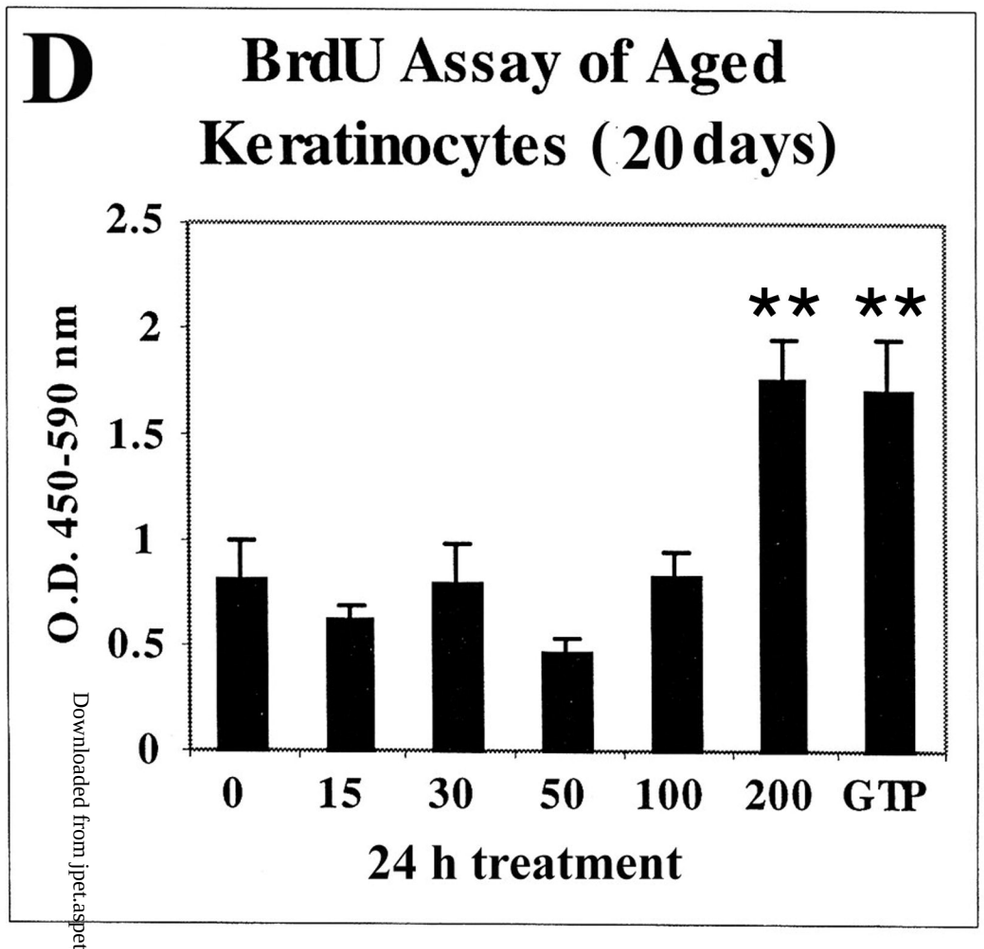


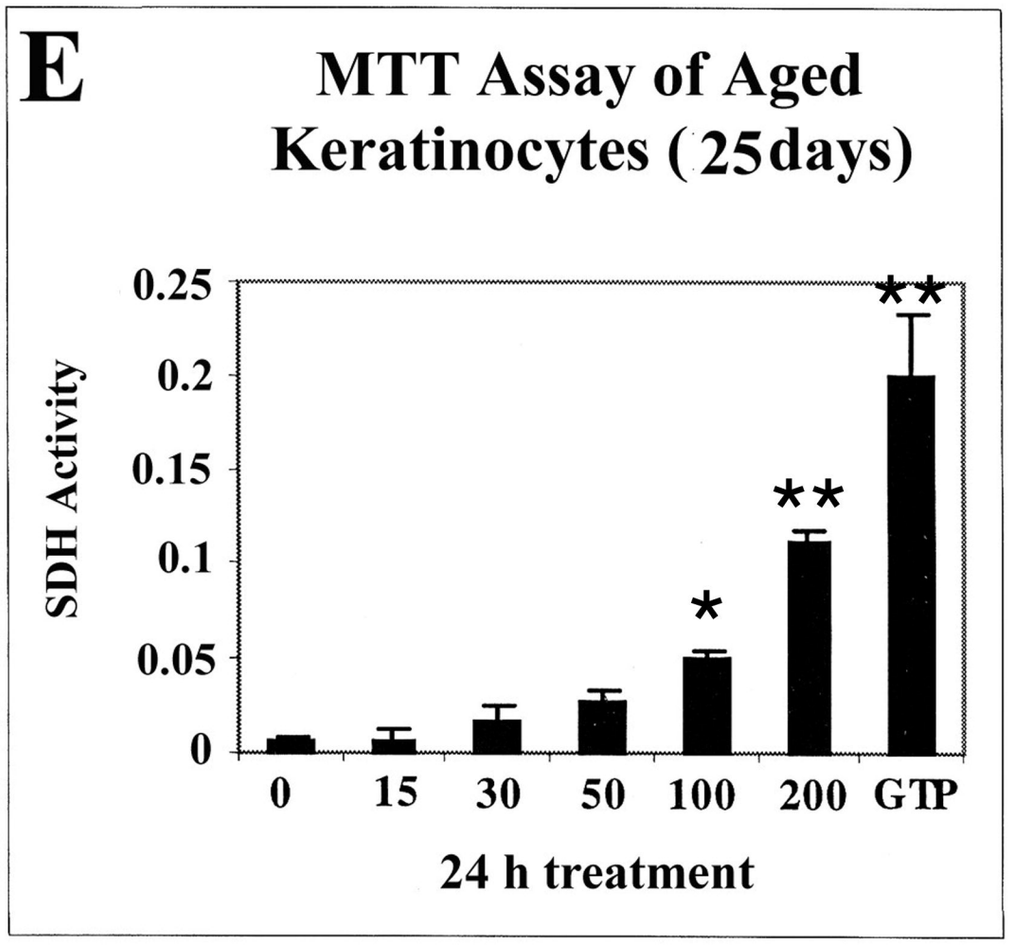
Fig. 3











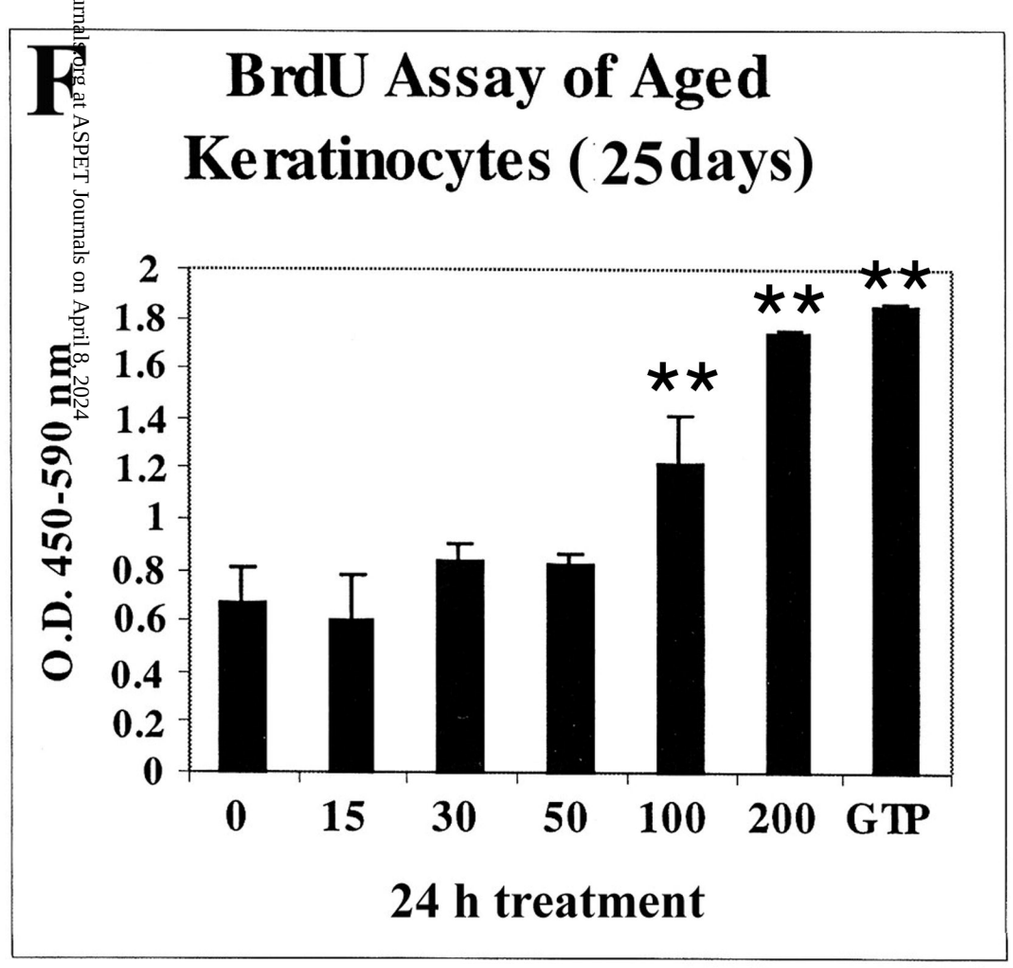


Fig. 4