Id-1 Delays Senescence But Does Not Immortalize Keratinocytes

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Running Title: Id-1 postpones but does not prevent senescence.

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Summary
Defining the molecular basis responsible for regulating the proliferative potential of keratinocytes has important implications for normal homeostasis and neoplasia of the skin. Under current culture conditions, neonatal foreskin-derived human keratinocytes possess a relatively short replicative lifespan. Recently it was reported that forced-overexpression of the helix-loop-helix protein Id-1, was capable of immortalizing keratinocytes, secondary to activation of telomerase activity and suppression of p16/Rb-mediated growth arrest pathways. To investigate the relationship between Id-1, telomerase activity, telomere length, p16, Rb cell cycle regulators, and senescence, whole populations of keratinocytes were infected with a retrovirus to induce overexpression of Id-1. In these unselected cultures, enhanced Id-1 levels clearly extended the lifespan of keratinocytes, but Id-1 did not prevent the onset of replicative senescence. Under these experimental conditions, Id-1 expression did not trigger induction of telomerase activity, and there was progressive shortening of the telomeres that was accompanied by elevated p16 levels, and prevalence of active Rb. The ability of Id-1 to postpone, but not prevent, senescence may be related to partial inhibition of p16 expression, as the Id-1 overexpressing cultures displayed a decreased capacity for TPA-mediated p16 induction. Thus, while no immortalization was observed, Id-1 could delay the onset of replicative senescence in unselected human keratinocyte populations.

Introduction
While studies examining replicative senescence have traditionally involved dermal fibroblasts as pioneered by Hayflick (1), more recently a growing interest in human keratinocyte senescent pathways has emerged (2-4). Immortalization of keratinocytes requires a genetically-defined program distinct from other cell types. For example, to achieve immortalization of keratinocytes requires inactivation of Rb/p16 function plus activation of telomerase, whereas in human fibroblasts, telomerase activation suffices to significantly extend their life span (5). Recently, it has been suggested that overexpression of the helix-loop-helix protein Id-1, was sufficient to immortalize primary human keratinocytes (6). It was concluded that Id-1 expression lead to activation of telomerase activity with inactivation of Rb and p16 in keratinocytes (ibid). Since the methodology employed in this report involved a selection step following transfection of the Id-1 cDNA, it was possible that the selected clones of immortalized keratinocytes had acquired other mutations that were contributing to bypassing of replicative senescence (4).

To further investigate the role of Id-1 in an unselected population of cultured keratinocytes, a retroviral vector containing the Id-1 cDNA was used to examine the relationship between Id-1 expression and the resultant phenotype in neonatal foreskin-derived human keratinocytes. Retrovirus-mediated gene-transfer facilitates introduction of target genes with high efficiency, so that effects of the transduced gene can be examined in whole cell populations (7). In contrast to the initial report (6), the overexpression of Id-1 by keratinocytes using this different experimental approach delayed the onset of replicative senescence, but did not prevent senescence. The Id-1 expressing keratinocytes with delayed senescence in later passaged cells resembled spontaneously senescent keratinocytes in that there was no increase in telomerase, but there were shortened telomeres (approximately 6kb in length), and increased p16, and active Rb levels. The importance of these findings is discussed in terms of normal epidermal homeostasis and cutaneous oncology (8-9).

Materials and Methods
1. Cell Culture and Treatment

Keratinocyte cultures were initiated from discarded neonatal foreskins, in which the epidermis was initially separated from dermis by exposure to dispase. The subsequent epidermal sheet was treated with trypsin / EDTA to produce a single cell suspension of keratinocytes that were placed in plastic petri dishes (Corning, NY) as previously described (3). Keratinocytes were fed with a low calcium (0.07 mm), serum-free medium (KGM, Clonetics Corp., San Diego, CA). From each foreskin, the initial epidermal suspension was seeded into 3 different 35 mm dishes, and then transferred to a 6-well cluster plate for retroviral infection. After infection and washing, keratinocytes were routinely cultured in 10 cm dishes, and passaged at 60-70% confluency with feeding using fresh KGM every other day. Cell counts to determine population doublings was performed manually using a hemocytometer and estimated based on the log₂ (number of keratinocytes at subculture/ number of cells initially plated) as primarily described (4). For induction of p16 and p21, representative keratinocytes from various passages were incubated with phorbol ester (TPA;100 nM; Sigma Chemical Co., St. Louis, MO) for 3 days, and then protein extracts performed as described below. For growth control and TPA response controls, neonatal foreskin-derived keratinocytes were derived exactly as above except they were either; never exposed to the retroviral infection, or were exposed to retroviral preparations containing only linker, but not containing the Id-1 cDNA.

2. Retroviral vectors and transduction of normal keratinocytes.

Id-1 cDNA was obtained from BamH1 and NOT1 of LZRS and MGF-based retroviral expression vector. The LZRS vector was kindly provided by Dr. Paul A. Khavari (Stanford University School of Medicine, Stanford CA) and used as previously described (10). The Phoenix-Ampo retroviral packing cells were obtained from American Type Culture Collection (Manassas VA) with permission from Dr. Gary P. Nolan (Stanford University Medical Center, Stanford, CA). The packing cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies Inc., Grand Island, NY) and transfected with Id-1 containing vector or linker (without Id-1) vector by using CaCl₂ and 2 x HBSS. After overnight incubation, the cells were fed with fresh medium and incubated at 32°C for an additional 24-48 h. The supernatants were collected for subsequent infection of primary cultures of keratinocytes.
The normal keratinocytes were seeded into 6-well plates and infected with 300 ul of viral supernatant in the presence of 4 ug/ml hexadimethrine bromide (Polybrene) (Sigma H-9268) for 1 h at 32°C, then the supernatant was removed and replaced with fresh medium, incubated at 37°C in 5% CO₂ overnight. After being washed with PBS, the infected cells were propagated and treated with TPA. The overexpression of the Id-1 protein was detected with Western blot and immunocytochemistry. Immunostaining was performed using the SC-488 anti-Id-1 ab (Santa Cruz) combined with an avidin-biotin peroxidase detection system (Vectastain, Vector Labs, Burlingame, CA). Positive staining was accomplished using 3-amino-4-ethylcarbazole as the chromagen producing a positive red reaction with 1% hematoxylin as the counter-stain as described previously (11).

3. Western Blot Analysis.

Whole cell lysates were prepared to detect different proteins. In brief, keratinocytes were washed with phosphate buffered saline, and were incubated on ice for 15 minutes in CHAPS buffer (3) with a cocktail of protease inhibitors (Boehringer Mannheim). Cells were microcentrifuged and supernatants were saved and frozen at -80°C. Protein concentration of each sample was determined by Bradford assay (Bio-Rad).

30 ug of protein were loaded on 6-12.5% SDS-polyacrylamide gel, transferred to Immobilon-p (polyvinylidene difluoride) membrane and blocked in 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). The membrane was incubated with the primary antibody in 2.5% powdered milk in TBST, washed extensively with TBST and then incubated with 1:1500 diluted anti-rabbit or mouse horseradish peroxidase (Amersham Pharmacia Biotech). Proteins were visualized with ECL reagents (Amersham Pharmacia Biotech) according to manufacturer’s instruction. Loading of proteins to verify equivalent distribution of proteins in each well was confirmed by Ponceau S staining. Antibodies against; p21 (SC397R); p16 (SC-759); Id-1 (SC-488); pRB (SC-50) and cdk4 (SC-260) were purchased from Santa Cruz and used at 1ug/ml concentration. Other antibodies were obtained from the following produced against: Bcl-x (mAb 1513A) and cyclin D1 (clone DCS-6); purchased from Pharmingen, San Diego, CA, and actin (clone C4; ICN Biomedicals, Inc., Costa Mesa, CA).
4. Telomerase Assay and Telomere Length Determination

The assay for telomerase activity utilized the PCR-based, telomerase repeat amplification protocol (TRAP assay); (12). The estimation of telomere length was performed by hybridizing a 32P-labeled telomeric (TTAGGG)$_4$ repeat probe to Hinfl and RsaI-digested genomic DNA separated on 0.8% agarose gels as previously described (13). Radioactive signal was quantified using a FUJIFILM Fluorescent Analyzer (FLA-2000), and analyzed using NIH image software. Weighted averages of the distances of band migration were calculated using Microsoft Excel, and compared to DNA molecular weight standard to determine mean telomere length.
Results

I. Overexpression of Id-1 Extends Keratinocyte Lifespan

Neonatal foreskin-derived keratinocytes normally proliferate rapidly in KGM (0.07 mM calcium), dividing every 1-2 days for 3-4 passages (approximately 20 population doublings; PD), before undergoing irreversible growth arrest (i.e. replicative senescence). Using unselected cell populations infected with an empty (only linker cDNA) retroviral vector, similar kinetics and number of PD was observed. However, unselected keratinocyte populations infected with an Id-1 containing retrovirus had a significantly greater number of population doublings (greater than 60 PD in two of the three independently generated Id-1 overexpressing cell lines), compared to uninfected or linker-control keratinocyte populations. Id-1 overexpressing keratinocytes also proliferated rapidly (dividing every 1-2 days), but in contrast to the earlier report (6), all three independently transduced cell populations ultimately did undergo irreversible growth arrest. The onset of replicative senescence in the three different Id-1 infected keratinocyte cultures occurred at PD 84, 62, and 52, respectively.

Upon onset of senescence, the keratinocytes underwent a change in morphology in which the small cuboidal-shaped proliferating cells became enlarged and flattened acquiring cytoplasmic vacuoles. The senescent keratinocyte cultures failed to undergo further proliferation despite frequent changes of medium and addition of fresh KGM. While the senescent keratinocytes remained viable for weeks with continued replacement of KGM, they displayed progressive detachment from the plastic dish, and upon exposure to mild trypsin/EDTA displayed a low tendency to re-attach (data not shown).

The relative levels of Id-1 for the different keratinocyte populations reveal higher Id-1 protein levels in the transduced keratinocytes at early passages, compared to the control keratinocyte populations (Figure 1). Examination of cytospins of retroviral infected keratinocyte cell lines by immunohistochemistry revealed approximately 80% of the population overexpressed Id-1 (data not shown).

Figure 1 displays several control lanes including the relatively low Id-1 levels in normal uninfected keratinocytes that were allowed to reach confluency (lane 1), or that had undergone spontaneous replicative senescence (lane 2). Normal proliferating keratinocytes had slightly
increased Id-1 levels (lane 3), but keratinocytes infected with the Id-1 containing retrovirus had markedly elevated Id-1 levels during their proliferative stages (lanes 4,5).

II. Molecular Phenotype of Senescent Keratinocytes

While overexpression of Id-1 by proliferating retroviral infected keratinocyte cell lines in early (PD 6 and PD 9) was expected (Figure 2), the relative levels of Id-1 in these transduced keratinocytes at the onset of replicative senescence, was of interest. When non-transduced keratinocytes undergo spontaneous senescence either due to confluency or replicative exhaustion, Id-1 levels are barely detectable compared to early passage proliferating normal keratinocytes (Figure 1; lanes 1 and 2). Interestingly, compared to these growth arrested non-transduced keratinocytes, the senescent Id-1 transduced keratinocyte lines continue to overexpress Id-1, although the relative levels decreased slightly compared to the proliferating Id-1 transduced keratinocytes examined at earlier passages (Figure 1; lanes 6 and 7). There was either no change, or minimal change, in the levels of either Bcl-x or β-actin (Figure 1). Examination of Bcl-x levels was selected because of the ability of Bcl-x to influence resistance of keratinocytes to apoptosis (14).

Since we previously established that keratinocytes undergoing spontaneous replicative senescence strongly induced p16 levels (3), Western blot analysis was performed on the keratinocyte cell lines. Normal proliferating keratinocytes possess low to absent p16 and p21 levels (Figure 2; lane 1). Early (PD 3) and mid-passage (PD 7) Id-1 overexpressing keratinocyte lines had either none or barely detectable levels of p16, and variable levels of p21 (Figure 2; lanes 2,3). However, at the onset of replicative senescence there was an increase in the p16 levels in both Id-1 overexpressing cell lines examined (Figure 2; lanes 4 and 5). To explore the levels of expression for cyclinD1 and cdk4, Western blot analysis of extracts prepared from the aforementioned cells was performed. Under all of these conditions, there was only relatively minor changes in levels of both cyclinD1 and cdk4 (Figure 2; lanes 1-5). Thus, both early population doubling keratinocytes in either control or Id-1 transduced cells, as well as keratinocytes undergoing senescence did not consistently increase or decrease either cyclinD1 or cdk4 levels.

The relative levels and phosphorylation status of Rb revealed the presence of both
inactive and active forms with prevalence of inactive forms in proliferating normal keratinocytes (Figure 3; lane 1), and early and mid-passage keratinocytes infected to overexpress Id-1 (Figure 3). However, upon onset of replicative senescence; Id-1 expressing keratinocyte cell lines expressed higher levels of the active (hypophosphorylated) form of Rb (Figure 3; lanes 6 and 7).

III. Telomerase Activity and Telomere Length

As the initial report suggested a link between Id-1 and telomerase activity (6), the keratinocyte populations at various passages were assayed for telomerase activity. Even though an immortalized cell line (i.e. HaCaT cells), or normal keratinocytes infected with a hTERT containing retrovirus had elevated telomerase levels, no increase in telomerase activity was detected in any of the three different Id-1 overexpressing keratinocyte cell lines, at either early or late passages (Figure 4). To follow up this observation, telomere lengths were also determined, and it became clear that there was progressive telomere shortening in the Id-1 overexpressing keratinocyte cell lines (Figure 5). While proliferating keratinocytes had telomere lengths between 10-11 kb (Fig 5: lanes 1,2) at the onset of replicative senescence in normal keratinocytes (lane 3) or Id-1 overexpressing keratinocyte cell line #1 (lane 4- PD 83), cell line #2 (lane 5 - PD 62), and cell line #3 (lane 6 - PD 52), telomere length in these cultured keratinocytes had been reduced to approximately 6-8 Kb. The immortalized HaCaT cells had the shortest telomeres (lane 7, 2.6 Kb).

IV. Id-1 Overexpression Modulates p16 Levels

To begin to probe the potential mechanism by which Id-1 could delay senescence in our keratinocyte populations, the influence of Id-1 levels on p16 induction was determined. Exposure of proliferating keratinocytes to phorbol ester (i.e. TPA) induces irreversible growth arrest accompanied by increased p16 levels after 72 hours following TPA treatment (Figure 6 - left side panel; lanes 1 and 2). By contrast to the p16 induction in normal keratinocytes either following TPA treatment (left side panel, lane 2) or onset of replicative senescence (right side panel, lane 2), the Id-1 overexpressing keratinocyte cell lines (right side panel, lane 3) displayed markedly reduced p16 induction following TPA exposure (right side panel, lane 4) but the cells
still underwent cell cycle arrest. The changes in p21 levels under these same conditions was characterized by an increase in p21 in the Id-1 overexpressing keratinocyte cell line exposed to TPA (Figure 6 - right side panel; lane 4). Thus, while Id-1 overexpression inhibits p16 induction by TPA, it does not inhibit a similar induction of p21.
A growing body of evidence implicates Id proteins such as Id-1 as regulators of cell growth in numerous cell lineages (reviewed in 8). In many cell types, including the normal keratinocytes examined in this study, Id-1 expression is down-regulated upon withdrawal of growth factors or onset of senescence (16-19). The forced overexpression of Id-1 in unselected keratinocyte populations using a retroviral vector significantly impacted the behavior of the cells by delaying the onset of replicative senescence. However, in contrast with the earlier report (6), we did not observe an immortalized phenotype, induction of telomerase, or inactivation of Rb in three different independently created cell lines overexpressing Id-1. The most likely explanation for this discrepancy is that the previous investigators isolated selected clones that included keratinocytes with elevated telomerase activity and inactivated Rb/p16 pathway. We suspect that our keratinocyte cell lines succumbed to the onset of replicative senescence secondary to shortened telomeres, since no induction of telomerase activity was detected. It would appear for some type of fibroblasts, retinal pigment epithelial cells, mesothelial cells as well as keratinocytes, that replicative senescence is a telomere length-sensitive response, with the primary difference reflected by the ability of hTERT overexpression, by itself, to overcome senescence in fibroblasts and retinal pigment cells (2,4,5). Taken together, the results in this report are consistent with the notion that immortalization of human keratinocytes requires not only inactivation of Rb/p16, but also activation of telomerase (5,11,20).

Another contributing factor to the difference between Alani et. al (6) and the current results is the earlier report utilized distinctive growth media. Alani et. al (ibid) established their primary cultures and expanded the keratinocyte populations using GIBCO/BRL media, whereas we utilized Clonetics media. The formulation of these media may permit or facilitate the outgrowth of different types of keratinocytes. Immortalization studies with viral oncoproteins of normal human mammary epithelial cells highlighted the genetic requirements for immortalization of a given population of cells can be dramatically different depending on the experimental protocol and reagents (21). Since squamous epithelia such as skin contain cells with stem cell-like properties that are TRAP positive, the inability to detect telomerase activity...
under the current conditions may point to the importance of specific media since Alani et al. (6) did detect some level of TRAP activity in their control transfected/selected keratinocyte population. Despite these potentially important technical differences, the positive findings of this report should be emphasized, including the extension of keratinocyte lifespan due to Id-1 overexpression, and the mechanistic insights involved in the prolonged survival as regards the cdk inhibitor p16 by Id-1. Based on the Western blot analysis of cyclinD1 and cdk4 (Figure 2), it does not appear that Id-1 has any significant direct effect on these cell cycle regulators.

Even though late passage keratinocyte lines overexpressed Id-1 (as well as stable levels of cyclin D1 and cdk4), this did not abrogate the growth-suppressive function of p16 and abundance of active Rb accompanying the onset of replicative senescence. Thus, these results reinforce the notion that the enhanced p16 level (rather than absent or low cyclinD1/cdk4) is responsible for the relative paucity of phosphorylated Rb, and are consistent with the apparent inability of Id-1 (and Id-3, but not Id-2) to directly interact with Rb (22,23). Exactly how Id-1 overexpression could suppress the TPA-mediated induction of p16 is under investigation. Whether activation of protein kinase C is important in this keratinocyte response also requires further exploration (24). The growth arrest of the Id-1 overexpressing cells induced by TPA in the absence of p16 induction, may have been triggered by the enhanced levels of p21, consistent with our earlier observation (3).

In conclusion, these results indicate that Id-1 has important growth-regulatory effects on keratinocyte populations, including a delay in the onset of replicative senescence. However, since telomerase is not directly activated by Id-1, telomeres continue shortening in these unselected keratinocyte lines triggering the onset of senescence, which is accompanied by elevated p16 levels and activated Rb. Further studies are indicated to more clearly define the link between Id-1 and regulation of p16 gene expression, as such a line of inquiry could lead to better understanding of the biology of epidermal stem cells (25), as well as cutaneous neoplasia (8,9,26,27). Thus, as an important component of normal tissue turnover and regenerative repair processes, the proliferative potential of transiently amplifying progenitor cells committed to the epithelial lineage must be tightly controlled. In human skin, disease states which include high epithelial turnover as seen following thermal injuries or in psoriasis (28) are associated with a
remarkably low frequency of keratinocyte neoplastic transformation - particularly salient when compared to transformation observed in epithelial cells of other tissues (e.g. colonic epithelium in inflammatory bowel disease). Thus, the current results together with previous reports (2,4,5,6,8,9) suggest that the molecular underpinnings of keratinocyte immortalization are quite distinct. These data converge upon the p16/Rb regulatory complex as a crucial regulator of keratinocyte replicative senescence that must be inactivated for telomerase-dependent immortalization. It will also be of interest to determine the role of Id-1 in normal cutaneous homeostasis, and the target genes and binding partners for Id-1 in epidermal keratinocytes.

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**Figure Legend**

**Figure 1**: Analysis of Id-1, Bcl-x, and actin protein by Western blot analysis in growth arrested and proliferating keratinocytes. Lane 1, confluent keratinocytes; Lane 2, senescent keratinocytes - PD 12; Lane 3, proliferating keratinocytes - PD 3; Lane 4, Id-1 overexpressing keratinocytes - PD 3; Lane 5, Id-1 overexpressing keratinocytes -PD 8; Lane 6, Id-1 overexpressing keratinocyte line #1 - PD 84 (i.e. senescent cells); Lane 7, Id-1 overexpressing keratinocyte cell line #2 - PD 62 (i.e. senescent cells).

**Figure 2**: Expression of p16, p21, cyclinD1, cdk4 and β-actin protein levels in normal proliferating keratinocytes (PD 5 - Lane 1), and Id-1 transfected keratinocyte cell line #1 in early (PD 3 - Lane 2); mid (PD 8 - Lane 3), and in Id-1 transfected cell line #1 at senescence (PD 84 - Lane 4); and senescent cell line #2 (PD 62 - Lane 5).

**Figure 3**: Rb expression in by Id-1 transfected keratinocytes reveals co-presence of both inactive (ppRb) as well as active (pRb) forms of Rb in early passage (PD 5) proliferating keratinocytes (Lane 1), but in subsequent passages, the Id-1 overexpressing cell populations have predominantly the inactive form of Rb, until the onset of senescence, at which time the active form of Rb becomes more prevalent. Lane 2, Id-1 overexpressing keratinocyte cell line #1 at PD 3; Lane 3, Id-1 overexpressing keratinocyte line #2 at PD 3; Lane 4 - PD 5; Lane 5 - PD 14; Lane 6 - PD 81; Lane 7 - PD 83.
Figure 4: Telomerase activity assays reveal in normal keratinocytes infected with hTERT containing retrovirus, elevated telomerase levels (Lane 1), as well as in immortalized HaCaT cells (Lane 2). No telomerase was detected in the representative keratinocyte cell line infected by a Id-1 containing retrovirus at either PD 7 or PD 11 for cell line #1; Lanes 3,4 respectively.

Figure 5: Telomere length was determined by phosphoimager analysis and computer assisted program software using relevant standards. The calculated mean telomere lengths are as follows: Lane 1, normal proliferating PD 3 keratinocytes - 11 kb; Lane 2, normal senescent PD 5 keratinocytes - 10 kb; Lane 3, normal senescent PD 16 keratinocytes - 8 kb; Lane 4, Id-1 overexpressing senescent cell line #1, PD 83 - 7.5 kb; Lane 5, Id-1 overexpressing senescent cell line #2, PD 62 - 8 kb; Lane 6, Id-1 overexpressing senescent cell line #3, PD 52 - 6.0 kb; Lane 7, immortalized HaCaT cells - 2.6 kb.

Figure 6: Id-1 overexpression blocks TPA-induced p16, but not p21 induction. In normal keratinocytes TPA induces p16 expression after 72 hours of exposure (left side panel - Lane 1, untreated keratinocytes; Lane 2, TPA treated keratinocytes ). While normal proliferating keratinocytes do not express either p16 or p21 (right side panel, Lane 1), upon the onset of replicative senescence (PD 15) both p16 and p21 are induced (right side panel, Lane 2). Cultures of keratinocytes overexpressing Id-1 (PD 5) contain low p16 and p21 levels (Lane 3), and these cultures do not respond to TPA by increasing p16, but do increase p21 levels (Lane 4).
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