Short Communication

Up-Regulation of p21^{WAF1} by Phorbol Ester and Calcium in Human Keratinocytes through a Protein Kinase C-Dependent Pathway

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Terminal differentiation in a variety of cell types has been associated with p53-independent up-regulation of p21^{WAF1}. p21^{WAF1} mRNA and protein are expressed at low levels in normal human skin, but overexpression of p21^{WAF1} has been observed in differentiating keratinocytes in involved psoriatic epidermis and in human squamous cell carcinoma. In this study we investigated by immunohistochemistry and Western blotting whether calcium and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, well characterized differentiation signals, induce p21^{WAF1} in cultured normal human keratinocytes and whether induction of p21^{WAF1} in this system depends on protein kinase C activation or functional p53. Phorbol ester induced p21^{WAF1} expression, which was maximal at 4 to 8 h with reduction back to baseline by 24 to 48 h. In contrast, increasing the extracellular Ca²⁺ concentration from 70 μ mol/L to 1.5 mmol/L resulted in up-regulation of p21^{WAF1} expression with a slower time course, with peak induction at 18 to 24 h. No parallel increase in p53 expression was observed in normal human keratinocytes. Up-regulation of p21^{WAF1} was also observed in response to phorbol ester in HaCaT cells, which carry homozygous and inactivating mutations for p53. Induction of p21^{WAF1} by phorbol ester and Ca²⁺ was inhibited by the specific protein kinase C inhibitor Ro 31-8220. The results demonstrate a differential time course of p21^{WAF1} protein up-regulation in response to phorbol ester and Ca²⁺, signals that result in keratinocyte differentiation, and suggest that induction of p21^{WAF1} in differentiating human keratinocytes occurs through protein kinase C-dependent and p53-independent mechanisms. (*Am J Pathol* 1998, 153:39–45)

Epidermis is a stratified squamous epithelium composed predominantly of keratinocytes. These cells divide in the

lower basal layers and subsequently move upward through the epidermis and undergo a complex program of morphological and biochemical changes, including the sequential expression of differentiation-associated proteins.¹ These changes lead to the formation of terminally differentiated cornified cells within the stratum corneum that form the primary barrier of skin. Abnormalities in keratinocyte differentiation are observed in a number of skin diseases, including psoriasis² and nonmelanoma skin cancer.^{3,4} Furthermore, it has been hypothesized that resistance of initiated cells to differentiation signals promotes a growth advantage during epidermal carcinogenesis.^{5,6}

Although there are some important differences between cell culture and human skin *in vivo*, cultures of keratinocytes have proved useful in investigating cell differentiation and identifying regulatory mechanisms involved in this process. Studies on cultured keratinocytes and *in vivo* have shown that the concentration of extracellular calcium ([Ca²⁺]_o) and activation of the protein kinase C (PKC) signal transduction pathway are important regulators of keratinocyte differentiation.^{7–13} Increased [Ca²⁺]_o stimulates formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol and activates PKC in mouse keratinocytes,^{14,15} although whether activation of PKC is required for [Ca²⁺]_o-induced human keratinocyte differentiation remains to be established. Indeed, mouse skin differs from human skin in several respects, including epidermal thickness, number of epidermal cell layers, and density of hair follicles, and murine and human keratinocytes show different responses to 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent PKC agonist and calcium.¹ For example, induction of ornithine decarboxylase is observed in mouse skin *in vivo* and cultured mouse keratinocytes in response to TPA, whereas TPA suppresses ornithine decarboxylase ex-

Supported in part by British Skin Foundation Grant 148/97 (to NJR).

Accepted for publication April 14, 1998.

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pression and enzyme activity in cultured human keratinocytes.^{16,17}

Terminal differentiation is coupled to withdrawal from the cell cycle, and this process has been associated with increased expression of p21^{WAF1} in a variety of tissues *in vivo*, including muscle and gastrointestinal epithelia,^{18–21} and in several well defined differentiating systems *in vitro*, including HL-60 cells.^{22,23} p21^{WAF1} (also known as CIP1, SDI1, MDA6, or CAP20) is transcriptionally regulated by p53²⁴ and is a downstream mediator of p53-induced growth arrest after DNA damage²⁴ through its interaction with proliferating cell nuclear antigen, cyclins, and cyclin-dependent kinases.^{21,25–27} p21^{WAF1} may also be regulated through p53-independent pathways in response to diverse stimuli including growth factors,²⁸ and experiments in p53-null cells indicate that increased expression of p21^{WAF1} during terminal differentiation occurs through p53-independent pathways.²³ Although some agents such as TPA induce p21^{WAF1} and act as differentiation signals in several cell systems, including HL-60 cells^{22,23} and keratinocytes,^{29,30} cell type-specific mechanisms also appear to be operating. In myoblasts, for example, the myoD family of transcription factors regulates p21^{WAF1} promoter activity, whereas in mouse keratinocytes another protein, p300, is required.²⁹

We have previously reported on increased p21^{WAF1} mRNA and protein expression in suprabasal psoriatic epidermis,³¹ with virtually absent expression in the basal layer. In the same study, we also observed up-regulation of p21^{WAF1} mRNA and protein expression in skin *in vivo* in response to agents that induce hyperproliferation,³¹ although whether p21 is involved in regulating this hyperproliferative response is not clear. Overexpression of p21^{WAF1} has also been reported in the differentiating cells of human cutaneous squamous cell carcinomas.³²

In the present study we have investigated whether increased [Ca²⁺]_o and TPA, two well characterized differentiation signals for human keratinocytes, induce p21^{WAF1} in cultured normal human keratinocytes and whether alteration in p21^{WAF1} expression is dependent on PKC activation and/or functional p53.

Materials and Methods

Cell Culture

Cultures of human keratinocytes were established from normal skin obtained from plastic and pediatric surgical procedures, essentially as described.³³ Epidermis was separated from dermis after overnight incubation at 4°C in Dispase II (neutral protease) (Boehringer Mannheim) and an epidermal cell suspension prepared after incubation with 0.05% trypsin and 0.02% ethylenediaminetetra-

acetic acid at 37°C. Keratinocytes were cultured in T75 flasks in MCDB-153 serum-free medium (Sigma-Aldrich) with a calcium concentration of 70 µmol/L, supplemented with ethanolamine (6.1 µl/L), phosphoethanolamine (14 µg/ml), hydrocortisone (0.18 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), epidermal growth factor (10 ng/ml), penicillin G (5 IU/ml), streptomycin (5 µg/ml), and amino acids.³⁴ Keratinocytes were expanded by serial passage and used for experiments between passages 2 and 3. Spontaneously immortalized HaCaT cells, a kind gift from Dr. NE Fusenig (German Cancer Research Center, Heidelberg, Germany), were cultured in Dulbecco's modified Eagle's medium (Sigma Laboratories, Poole, UK) with 10% fetal calf serum.

Immunohistochemistry

Primary cultures of keratinocytes and HaCaT cells were passaged by trypsinization and were subsequently seeded into 12-well plates containing one round 16 mm-diameter coverslip/well at a density of 1 × 10⁵ cells/well. After timed incubation with specified agents or with medium containing 1.5 mmol/L Ca²⁺, cells were fixed in 4% paraformaldehyde and immunostained with anti-p21^{WAF1} monoclonal antibody, diluted 1:70 (Oncogene Research Products, Cambridge, MA), using an avidin-biotin immunoperoxidase kit (Vector Laboratories, Burlingame, CA), with Ni²⁺ plus 3,3'-diaminobenzidine as the chromagen and counterstained with methyl green.

Western Analysis

Cells were treated with the specified agents and scraped into 2× sodium dodecyl sulfate lysis buffer (125 mmol/L Tris-HCl, pH 6.8, 0.05% bromphenol blue, 4% sodium dodecyl sulfate, 20% glycerol, and 10% β-mercaptoethanol). Protein concentrations were determined on samples, before addition of β-mercaptoethanol, using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions. Equal protein quantities of samples were electrophoresed through 12.5% polyacrylamide gels, transferred onto nitrocellulose, blocked in blocking buffer (5% nonfat milk in Ca²⁺ and Mg²⁺-free PBS) for 1 hour at room temperature, and subsequently incubated overnight at 4°C in the appropriate dilution of primary mouse monoclonal antibody (anti-p21^{WAF1}, 1:250; DO7, anti-p53; 1:1000; Novocastra Laboratories, Newcastle, UK) in blocking buffer. After incubation with rabbit anti-mouse peroxidase-conjugated secondary antibody and avidin-biotin complex, membranes were washed and protein bands were visualized using an enhanced chemilumines-

Figure 1. Kinetics of increased p21^{WAF1} expression in human keratinocytes in response to TPA and increased extracellular calcium. **A** and **B**: Cultured human keratinocytes derived from the same donor were treated with TPA (50 nmol/L) (**A**) or switched to medium containing 1.5 mmol/L [Ca²⁺]_o (**B**) for various times (in hours) and immunostained with anti-p21^{WAF1} monoclonal antibody. Essentially identical results were obtained in at least three independent experiments from three different donors. **C** and **D**: Cultured human keratinocytes derived from the same donor were treated for the specified times with TPA (50 nmol/L) (**C**) or switched to medium containing 1.5 mmol/L [Ca²⁺]_o (**D**), and cell lysates were prepared, fractionated in sodium dodecyl sulfate/12.5% polyacrylamide gels, transferred to nitrocellulose membrane, and immunoblotted with anti-p21^{WAF1} monoclonal antibody, as described in Materials and Methods. **Lane 1**, vehicle; **lane 2**, 2 h; **lane 3**, 4 h; **lane 4**, 8 h; **lane 5**, 18 h; **lane 6**, 24 h; **lane 7**, 48 h. Relative densitometric values (arbitrary units) are indicated below the lanes.

Figure 2. Ro 31-8220 partially inhibits up-regulation of p21^{WAF1} by TPA and increased [Ca²⁺]_o. Cell lysates were prepared from cultured human keratinocytes treated with the specified agents, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with anti-p21^{WAF1} monoclonal antibody, as described in Materials and Methods. **A:** Lane 1, vehicle; lane 2, TPA (50 nmol/L) for 4 h; lane 3, TPA (50 nmol/L) plus Ro 31-8220 (100 nmol/L) for 4 h; lane 4, Ro 31-8220 (100 nmol/L). **B:** Lane 1, vehicle; lane 2, 1.5 mmol/L [Ca²⁺]_o for 8 h; lane 3, 1.5 mmol/L [Ca²⁺]_o plus Ro 31-8220 (100 nmol/L) for 8 h. Relative densitometric values (arbitrary units) are indicated below the lanes. Results are representative of at least three independent experiments that yielded similar results.

cent Western blotting detection system (Pierce). Band intensities were quantified by laser densitometry (LKB model 2222-020, LKB Prodakter, Bromma, Sweden) according to the manufacturer's instructions.

Results

Human keratinocytes cultured as monolayers in low-calcium (70 μ mol/L), serum-free MCDB-153 medium differentiate in response to TPA and increased extracellular calcium [Ca²⁺]_o (1.5 mmol/L).^{8,10,12,13} We initially investigated changes in p21^{WAF1} protein expression by immunohistochemistry in human keratinocytes after exposure to TPA and increased [Ca²⁺]_o. Figure 1A shows that compared to control, TPA (50 nmol/L) induced p21^{WAF1} expression in cultured human keratinocytes with maximal induction at 4 to 8 h and reduction back toward baseline at 24 to 48 h. As expected, p21^{WAF1} expression was

predominantly nuclear. In parallel experiments on keratinocytes derived from the same donor, increased p21^{WAF1} expression was also observed in response to increased [Ca²⁺]_o but with a slower time course compared to TPA (Figure 1B), and substantial expression persisted at 24 and 48 h. Induction of p21^{WAF1} expression after treatment of paired human keratinocyte cultures with TPA and increased [Ca²⁺]_o was confirmed by Western analysis (Figure 2, A and B). Western blotting experiments also confirmed a differential time course of p21^{WAF1} induction in response to TPA and increased [Ca²⁺]_o (Figure 1, C and D), with maximal induction at 4 to 8 h and 18 h for TPA and increased [Ca²⁺]_o, respectively.

Because PKC agonists induce keratinocyte differentiation and because PKC-mediated cell cycle arrest involves induction of p21^{WAF1} in a number of cell types, including HL-60 cells and intestinal epithelial cells,^{23,35} we investigated the effect of the relatively specific PKC inhibitor Ro 31-8220³⁶ on p21^{WAF1} expression in differentiating human keratinocytes. Western analysis showed that Ro 31-8220 (100 nmol/L) substantially abrogated TPA-induced up-regulation of p21^{WAF1} at 4 h and 1.5 mmol/L [Ca²⁺]_o-induced up-regulation of p21^{WAF1} at 8 h (Figure 1, C and D). Similar results were obtained using immunohistochemical analysis (data not shown). No significant induction of p21^{WAF1} was observed after exposure of keratinocytes to Ro 31-8220 alone (Figure 2A). These results provide evidence that early up-regulation of p21^{WAF1} in human keratinocytes in response to both TPA and increased [Ca²⁺]_o occurs through a PKC-dependent pathway.

p53-null mouse keratinocytes retain their ability to respond to differentiation signals and to up-regulate p21^{WAF1} in response to increased [Ca²⁺]_o.^{29,30,37} In our experiments in which TPA and increased [Ca²⁺]_o resulted in up-regulation of p21 in normal human keratinocytes, we observed a progressive reduction in p53 ex-

Figure 3. Induction of p21^{WAF1} in human keratinocytes is independent of p53. **A and B:** Cultured human keratinocytes derived from the same donor were treated with the specified agents, and Western analysis of cell lysates was performed using an anti-p53 monoclonal antibody. **A:** Lane 1, vehicle; lane 2, TPA (50 nmol/L) for 2 h; lane 3, TPA (50 nmol/L) for 4 h; lane 4, TPA (50 nmol/L) for 8 h; lane 5, TPA (50 nmol/L) plus Ro 31-8220 (100 nmol/L) for 8 h; lane 6, TPA (50 nmol/L) for 18 h; lane 7, TPA (50 nmol/L) for 24 h; lane 8, TPA (50 nmol/L) for 48 h. **B:** Lane 1, 70 μ mol/L [Ca²⁺]_o; lane 2, 1.5 mmol/L [Ca²⁺]_o for 2 h; lane 3, 1.5 mmol/L [Ca²⁺]_o for 4 h; lane 4, 1.5 mmol/L [Ca²⁺]_o for 8 h; lane 5, 1.5 mmol/L [Ca²⁺]_o plus Ro 31-8220 (100 nmol/L) for 8 h; lane 6, 1.5 mmol/L [Ca²⁺]_o for 18 h; lane 7, 1.5 mmol/L [Ca²⁺]_o for 24 h; lane 8, 1.5 mmol/L [Ca²⁺]_o for 48 h. **C and D:** Cultured HaCaT cells containing functionally inactive homozygous mutations of p53 were treated with TPA (50 nmol/L) for 4 h (lane 2) or 24 h (lane 3) or treated with vehicle (lane 1), and Western analysis of cell lysates was carried out using anti-p21^{WAF1} (C) and anti-p53 monoclonal antibodies (D). Relative densitometric values (arbitrary units) are indicated below the lanes.

pression after treatment with TPA (Figure 3A) but no significant change in p53 expression in response to increased $[Ca^{2+}]_o$ (Figure 3B). Interestingly, TPA-induced down-regulation of p53 was substantially reduced by Ro 31-8220 (Figure 3A). As p53-null human keratinocytes are not currently available for study, we used HaCaT cells (a spontaneously immortalized human keratinocyte cell line) containing two mutant alleles of p53,³⁸ which are unable to transactivate p21,³⁹ to examine whether p53 is required for p21^{WAF1} induction in human keratinocytes. Induction of p21^{WAF1} in HaCaT cells was observed by Western analysis (Figure 3C) and immunohistochemistry (data not shown), indicating that p53 is not required for this response. No reduction in basal p53 protein expression was observed in HaCaT cells in response to TPA (Figure 3D).

Discussion

In this study we have examined the relationship between two well characterized keratinocyte differentiation signals, increased $[Ca^{2+}]_o$ and TPA, and p21^{WAF1} expression in cultured normal human keratinocytes. We have used a specific PKC inhibitor and HaCaT cells to determine whether induction of p21^{WAF1} by increased $[Ca^{2+}]_o$ and TPA requires PKC activation or functional p53. Our results demonstrate a low level of p21^{WAF1} protein expression in proliferating cultured human keratinocytes consistent with virtually absent p21^{WAF1} mRNA and protein expression in normal human skin.³¹ Calcium-induced differentiation in mouse keratinocytes has previously been associated with up-regulation of p21^{WAF1}.^{29,30} We observed up-regulation of p21^{WAF1} in human keratinocytes in response to TPA and increased $[Ca^{2+}]_o$. Both TPA and increased $[Ca^{2+}]_o$ induce growth arrest and terminal differentiation in human keratinocytes.^{7,8,10,12} However, TPA induces more rapid and profound differentiation than calcium in these cells.⁴⁰ Our experiments show a differential time course of p21^{WAF1} up-regulation, with TPA inducing p21^{WAF1} protein more swiftly than increased $[Ca^{2+}]_o$. These results further support an association between induction of p21^{WAF1} and terminal differentiation in human keratinocytes. p21^{WAF1} expression appears low in normal skin as detected by immunohistochemistry and *in situ* hybridization, and it is therefore difficult to comment about the relative expression of p21^{WAF1} in basal and suprabasal differentiating keratinocytes.³¹ However, increased p21^{WAF1} mRNA and protein was observed in suprabasal differentiating keratinocytes in involved psoriatic epidermis,³¹ which displays abnormal keratinocyte differentiation.² Similarly, in cutaneous human squamous cell carcinomas, which show alterations in keratin expression compared to normal epidermis,^{3,4} p21^{WAF1} is overexpressed in superficial differentiating keratinocytes.³² Further studies are required to determine whether these observations reflect a role for p21^{WAF1} in induction and maintenance of normal keratinocyte differentiation *in vivo* or whether p21^{WAF1} accumulates because of an abnormal differentiation program.

The bisindolylmaleimide Ro 31-8220 is a relatively specific PKC inhibitor³⁶ that inhibits TPA-induced growth arrest and differentiation in human keratinocytes.⁴¹ Our studies with Ro 31-8220 provide important evidence that induction of p21^{WAF1} protein by both TPA and increased $[Ca^{2+}]_o$ in human keratinocytes is dependent in part on PKC activation. In human keratinocytes, increased $[Ca^{2+}]_o$ results in inositol 1,4,5-trisphosphate formation⁴² and increased intracellular Ca^{2+} ($[Ca^{2+}]_i$),⁴³ but whether increased $[Ca^{2+}]_o$ activates PKC in human keratinocytes remains to be formally established. We are currently performing experiments to address this issue. Up-regulation of p21^{WAF1} by PKC activation in human keratinocytes may involve the Sp family of transcription factors, as TPA significantly increased Sp1 protein levels and Sp1 binding in K562 cells,⁴⁴ and Sp1 and Sp3 both activate the p21^{WAF1} promoter, although overexpression studies suggest that only Sp3 is directly involved in promoter induction during mouse keratinocyte differentiation.⁴⁵

Although up-regulation of p21^{WAF1} is associated with cellular differentiation, p21^{WAF1}-null mice showed normal organ and skin development with no morphological defects in cell differentiation, indicating that p21^{WAF1} is not essential.^{46,47} However, primary keratinocytes derived from p21^{WAF1}-null mice showed a significantly increased proliferative potential and an alteration of the keratinocyte differentiation program with a reduction of late differentiation markers.⁴⁷ In addition, the absence of p21^{WAF1} resulted in aggressive keratinocyte-derived tumors after transformation by *ras*.⁴⁷ These results and our previous work³¹ suggest that p21^{WAF1} may be involved in the regulation of keratinocyte differentiation and that dysregulation of p21^{WAF1} expression in human skin may contribute to abnormal keratinocyte differentiation observed in psoriasis and cutaneous squamous cell carcinoma.

Up-regulation of p21^{WAF1} in HaCaT cells by transforming growth factor β , which induces cell cycle arrest but not cell differentiation, indicates that induction of p21^{WAF1} is not sufficient for keratinocyte differentiation.³⁹ Datto et al. also showed that HaCaT cells contain two mutant p53 alleles that are unable to transactivate the p21^{WAF1} promoter when overexpressed, indicating that induction of p21^{WAF1} by transforming growth factor β occurs through a p53-independent mechanism.³⁹ Within lesional psoriatic epidermis in which p21^{WAF1} was overexpressed in suprabasal layers, p53 protein expression was only marginally elevated within the low epidermal layers, suggesting that induction of p21^{WAF1} in psoriatic epidermis may be independent of p53.³¹ Our observations in human keratinocytes and HaCaT cells support this hypothesis. A decrease in p53 protein expression was observed in response to TPA (Figure 3A) consistent with experiments in mouse keratinocytes.³⁰ Interestingly, this response was almost completely blocked by Ro 31-8220 (Figure 3A). However, our findings of stable p53 protein expression in human keratinocytes after treatment with increased $[Ca^{2+}]_o$ (Figure 3B) contrasts with reduced p53 synthesis in mouse keratinocytes.³⁰ Although the absence of increased p53 protein expression in response to TPA and increased extracellular Ca^{2+} (Figure 3, A and B)

does not exclude a p53-mediated response,³⁰ induction of p21^{WAF1} by TPA in HaCaT cells provides evidence for a p53-independent mechanism of action in human keratinocytes, consistent with observations in p53-null mouse keratinocytes.²⁹

Three main conclusions can be drawn from the results presented. TPA and increased $[Ca^{2+}]_o$ induced up-regulation of p21^{WAF1} in human keratinocytes with different time courses. Up-regulation of p21^{WAF1} by both TPA and increased $[Ca^{2+}]_o$ in human keratinocytes is dependent in part on PKC activation. Finally, up-regulation of p21^{WAF1} by TPA occurs through a p53-independent mechanism.

Acknowledgments

We thank Dr Eugene Healy and Professor Jonathan Rees for critical review of the manuscript and helpful suggestions.

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