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Molecular characterization and expression of p63 isoforms in human keloids

Abstract Keloids are benign skin tumors that develop following wounding. A cDNA product from human keloid specimens was identified using the differential display technique. The full-length cDNA was cloned by RT-PCR using human keloid mRNA as template. The predicted product of the cDNA was found to be 99% identical to the ΔN-p63 gamma isotype of p63, a transcription factor that belongs to the family that includes the structurally related tumor suppressor p53 and p73. The ΔN-p63 isotype lacks the acidic N terminal region corresponding to the transactivation domain of p53. Since this can potentially block p53-mediated target gene transactivation, it may serve as a dominant-negative isoform. Real-Time RT-PCR analysis of RNAs from normal skin tissue and keloids showed that the ΔN-p63 isotype is specifically expressed in keloids, but is virtually undetectable in normal skin. Immunostaining of p63 in normal skin revealed that only basal cells of the epithelium expressed the protein, while in keloid tissues the antigen was detected in the nuclei of cells scattered through all layers of the epithelium and in fibroblast-like cells in the dermis. These results may indicate that aberrant p63 expression plays a role not only in malignant tumors but also in benign skin diseases that show hyperproliferation of epidermal cells in vivo. Moreover, this isoform of p63 could serve as a specific molecular marker for this human disease.

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Introduction

Keloids are fibrous overgrowths that develop at sites of cutaneous injury and, unlike normal scars, do not regress. Although the cause of keloids is unknown, it is thought that they are due to a failure to turn off the healing process needed to repair broken skin. When this occurs, extra collagen forms at the site of the scar, and continues to form because the process is not shut off. This results in keloid formation. Black Africans form keloids more easily than Caucasians.

Keloids are characterized by the formation of excessive scar tissue, which proliferates beyond the boundaries of the original wound. They are considered to be benign tumors, but they never become malignant. They represent a model for aberrant wound healing characterized by alterations in apoptosis and cell proliferation.

It is now accepted that apoptosis, or programmed cell death, plays a pivotal role during embryogenesis, tissue remodeling and cell turnover. Apoptosis is triggered by genes that code for apoptosis mediators, including the tumor suppressor p53 (Weedon 1990; Paus et al. 1995; Desmouliere et al. 1995). Expression of the proteins Bcl-2, c-Jun and c-Fos in fibroblast-like and perivascular spindle cells is known to be related to increased fibroblast proliferation in keloids. Such cells also stain positively for Ki67, a nuclear antigen associated with cell proliferation, which is present throughout the active cell cycle (G1, S, G2 and M phases) but is absent from resting cells (G0). These observations suggest that pathological scarring may result from alterations in genes that would normally induce apoptosis (Teofoli et al. 1999).

Determination of the proliferation index using the Ki67 antigen as a marker gives an accurate indication of the incidence of proliferating cells, and there is a strong

correlation between low and high Ki67 index and low- or high-grade histopathology of neoplasms (Gerdes et al.1983, 1991).

Also, low rates of apoptosis, and low levels of p53 expression, have been detected that could account for the dysregulated growth documented in keloids (Ladin et al. 1998).

In the present study, we used differential display analysis to identify and characterize keloid-specific genes in humans. We have characterized one keloid-specific transcript, which codes for the ΔN-p63 gamma isotype of p63. p63 is a member of the same family of transcription factors as p53 and p73. However, the ΔN gamma isoform of p63 lacks the N-terminal transactivation domain, and it has been shown to have a dominant-negative effect upon p53 in vitro, preventing the latter from activating its target genes.

Transactivating (TA) isoforms of p63 are also known. In contrast to the ΔN-p63 gamma isotype, these contain a p53-homologous N-terminal transactivating domain that can activate p53 promoter-reporter genes and induce apoptosis (Yang et al. 1998, 1999). Thus, the tissue distribution of the p63 isoforms may be relevant to the regulation of p53 in vivo. In normal epidermis, hair follicles, and stratified squamous cell cultures, the ΔN-p63 gamma form of the p63 protein is restricted to cells with a high proliferative potential and is absent in cells undergoing terminal differentiation (Parsa et al. 1999).

Studies on p63 overexpression in tumor cells support the idea that these proteins play an important role in the development of human primary tumors, including squamous and transitional cell carcinomas (Nishi et al. 1999; Park et al. 2000; Yamaguchi et al. 2000) and in benign salivary gland tumors (Friedman et al. 1993).

In addition, these studies support the concept of a neoplastic and proliferative potential of aberrant p63/p73 expression even in nonmalignant tumors. In particular, previous studies have suggested that overexpression of ΔN-p63 may be oncogenic, because of its ability to block the activity of p53 (Yang et al. 1998; Crook et al. 2000; Liefer et al. 2000). Indeed, ΔN-p63, the predominant isotype in proliferating epithelium, is overexpressed in squamous cell carcinomas (Crook et al. 2000; Hu et al. 2002).

Here, we report on the cellular localization and differential expression of the ΔN-p63 isoform in keloids and in normal skin, and its possible role in the development of keloids is discussed.

Materials and methods

Tissue collection

Skin specimens from ten clinically active keloids (active for more than 1 year) and ten healthy controls were surgically removed, frozen and stored at –80°C prior to use.

RNA isolation

Total RNAs were extracted from normal human epidermis and human keloid tissue samples with the High Pure RNA isolation kit (Roche), according to the manufacturer's instructions. Traces of contaminating DNA were removed by treatment with DNase I.

Differential display analysis

Differential display analysis was performed according to the method of Liang and Pardee (1992) with slight modifications. Briefly, the anchored primers 5′-(T)$_{11}$ G-3′, 5′-(T)$_{11}$ A-3′ and 5′-(T)$_{11}$ C-3′ were used with five arbitrary 13mer primers (5′-CTGCTTTGAGGCC-3′, 5′-GCTCTACCACCTT-3′, 5′-CCTGAGCCCGCCG-3′, 5′-CATGATGGTCAAG-3′ and 5′-CACCCTCATCGTC-3′).

First-strand cDNA was synthesized using AMV reverse transcriptase with each anchored primer, and with total RNA as template. The single-stranded cDNA was then used as a template for PCR with the five arbitrary primers, respectively. To rule out genomic DNA contamination, we routinely included a negative control containing RNA instead of cDNA.

Aliquots of the PCR products were subjected to electrophoresis on a 3% agarose gel. Bands that appeared to be up- or down-regulated in keloid tissues were cut out of the gels with razor blades, and the DNA was eluted and directly cloned in the pDrive vector (Promega).

The cDNAs were sequenced using an ABI PRISM 310 Genetic Analyzer (Hewlett-Packard), according the manufacturer's instructions.

Homology searches in the GenBank and EMBL databases were performed using BLAST (Altschul et al. 1990) and FASTA (Pearson et al. 1988).

Isolation of full-length cDNAs

cDNAs were synthesized from poly(A)$^+$ RNA isolated from human keloid specimens using the Marathon cDNA amplification kit. This cDNA mixture was then used as the template for PCR amplification with sense and antisense primers specific for ΔN-p63 gamma [5′-ATGTTGTACCTGGAAAACAATGCCC-3′ (nt 1–25) and 5′-TTTGGCTAGTCACATGGGTATC-3′ (nt 1161–1182), respectively]. A full-length cDNA for ΔN-p63 gamma was obtained and cloned into pT-Adv. The sequence of the sense primer was based on the results of 5′RACE analysis of part of the ΔN-p63 cDNA fragment isolated by differential display.

DNA sequencing

To check the specificity of the amplified products, DNA bands were eluted from gels, purified and sequence

analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 Perkin-Elmer).

Quantitative Reverse Transcription PCR (Real-Time PCR)

cDNAs for p63 isoforms were synthesized and amplified from 100-ng aliquots (1 µl) of total RNA in a reaction mixture containing 5 pmol of the specific primer pair TAp63 sense (5′-GTCCCAGAGCACACAGACAA-3′) and TAp63 antisense (5′-GAG-GAGCCGTTCTGAATCTG-3′, or ΔN-p63 sense (5′-CAGACTCAATTTAGTGA-3′) and ΔN-p63 antisense (5′-AGCTCATGGTTGGGG CAC-3′), 7.5 µl of a 2.7× Cycler RNAMasterSYBR Green I solution (Roche) and 1.3 µl of manganese acetate stock solution. The mixture was incubated for 30 min at 50°C and the cDNA product was amplified by 40 cycles of PCR (30 s at 94°C, 30 s at 57°C, and 1 min at 72°C). The incorporation of the dye into the amplified products was monitored with Light-Cycler (Roche), and the concentration of a specific transcript in the sample was analyzed, based on predetermined standard curves constructed with known amounts of target transcripts, using the associated software. Transcripts of the β-actin gene were used as a total-cDNA control. Results from three independent RT-PCR analyses were averaged.

RT-PCR

cDNAs for p53 were synthesized and amplified from total RNA (1 µg) using the RT-PCR Beads kit (Amersham Biosciences) according to the manufacturer's protocol using the p53 isoform-specific primers p53 sense (5′-ATGTTTTGCCAACTGGCC-3′) and p53 antisense (5′-AGGCTCCCCTTTCTTGCG-3′).

All reverse transcription reactions were carried out for 30 min at 60°C and then incubated for 3 min at 94°C, followed by PCR conditions for 40 cycles of 95°C for 45 s, 57°C for 1 min and 72°C for 2 min. The samples were then incubated for an additional 7 min at 72°C.

Transcripts of the β actin gene were chosen to test the integrity of the RNA in the RT-PCR assay, and these were amplified using forward 5′-CCAAGGCCAACCGCGAGAAGATGAC-3′ and reverse (5′-AGGGTACATGGTGGTGCCGCCAGAC-3′) primers. RT-PCRs were performed in triplicate (including actin and a one-primer control), and a 25-µl aliquot was electrophoresed in 1% agarose gels.

Immunocytochemistry

Tissues were fixed by immersion in Bouin's fluid for 24 h at room temperature or in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4) for 12 h. After immersion for 5 days in 75% alcohol, the tissues were embedded in 56–58°C paraffin (Paraplast). Serial, paraffin-embedded sections 7 µm thick, were mounted on slides coated with albumin glicerinate, and processed for the immunohistochemical localization of p63 immunoreactivity. Briefly, deparaffinized sections were rinsed in PBS and sequentially incubated in normal goat serum (NGS, diluted 1:50; Pierce) for 20 min to reduce undesired background staining, primary antibody (monoclonal mouse anti-p63, diluted 1:1000; Santa Cruz Biotechnology) overnight at 4°C in a dark moist chamber, biotinylated secondary antibody (goat anti-mouse IgG, diluted 1:200; Pierce) for 1 h at room temperature, and streptavidin (diluted 1:200; Pierce) for 1 h at room temperature. Then the immunoreaction was visualized by exposure to 3.3-diaminobenzidine tetrahydrochloride (in TRIS buffer; Sigma) and H_2O_2. The specificity of the immunostaining was demonstrated by (1) omission of the primary antibody, (2) replacement of the primary antibody by NGS or PBS. No immunoreactivity was detected in any of these control tests.

Results

Isolation and characterization of a cDNA clone encoding ΔN-p63 gamma

Gene expression in human keloids and normal skin specimens was analyzed by the differential display method. Differential display patterns obtained with one of 15 combinations of the anchored oligo-dT primer and an arbitrary primer [5′-(T)$_{11}$ C-3′ and 5′-CTGCTTTGAGGCC-3′] revealed four cDNAs which differed significantly in amount between the two different specimens. Three of these, however, were derived from mRNAs for proteins which are known to be expressed in various tissues and were not considered to be keloid-specific. The predicted products of these cDNAs included ferritin and polygalacturonase. The remaining cDNA of about 250 bp in length was detected clearly in keloid specimens but was scarcely detectable in normal skin (Fig. 1).

When we searched the GenBank database using the BLAST program with the nucleotide sequence of this cDNA as the query, it was found to be similar to that of ΔN-p63 gamma isotype (Accession No. AF075429). Although the cDNA contained only the 3′ half of the full-length cDNA, the 5′ coding and flanking regions were obtained using the 5′RACE method. The full-length cDNA was finally cloned by RT-PCR from mRNA isolated from keloid specimens, using appropriate primers, and was found to show 99% identity to the cDNA for ΔN-p63 gamma protein (AF075429).

Expression of p63 and p53 RNA in skin tissue

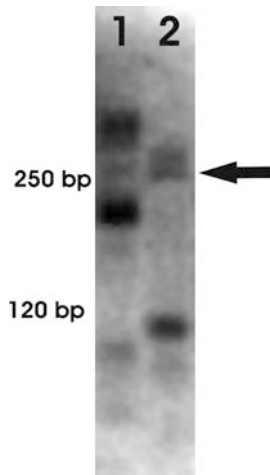The expression of ΔN-p63 and the longer transactivating forms (TA-p63) of p63 in both normal skin tissue and

Fig. 1 Differential display analysis of gene expression in normal human skin (lane 1) and human keloid specimens (lane 2). The *arrow* indicates the band corresponding to the cloned (ΔN-p63 gamma) cDNA fragment of 250 bp

Fig. 3A, B RT-PCR analysis of p53 expression in keloids (**A**) and normal skin (**B**). **A** Left lane, 100-bp molecular weight marker (Amersham Pharmacia); lane 1, p53 fragment (520 bp); lane 2, β-actin fragment (587 bp); lane 3, negative control using p53 primers without RNA. **B** Left lane, 100-bp molecular weight marker (Amersham Pharmacia); lane 1, β-actin fragment (587 bp); lane 2, negative control using p53 primers without RNA. lane 3, p53 fragment (520 bp)

keloids was analysed by Real-Time RT-PCR. Using this assay, the levels of TA-p63 and ΔN-p63 RNAs in normal tissue and keloids were compared. ΔN-p63 was reproducibly found to be more abundant in the keloid specimens, and the signal obtained from normal skin tissue was quite weak. This is consistent with the results of the differential display analysis. Moreover, there was no significant difference in TA-p63 expression between the two tissues (Fig. 2).

Conventional RT-PCR was used to assay for the presence of p53 in keloid specimens. while β actin gene expression was chosen to test the RNA integrity in RT-PCR assay (Fig. 3). The results indicated that p53 is expressed at very low levels in keloid tissue relative to normal skin, in agreement with the findings of Ladin et al. (1998).
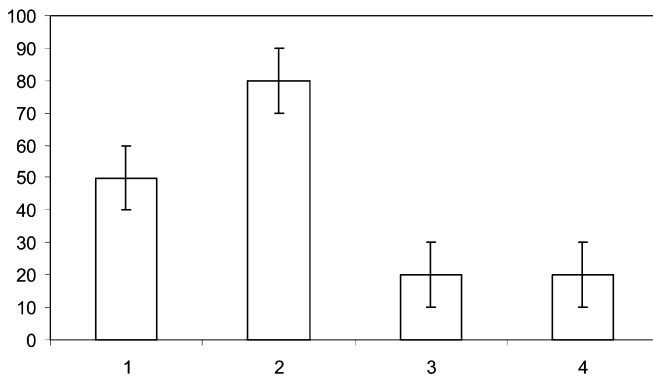
Immunohistochemistry

Immunohistochemical analysis of human normal skin revealed that basal cells of the epithelium (Fig. 4) were positive for p63, as has been reported previously for other epithelial sites (Yang and McKeon 2000). In contrast, human keloid tissues showed strong p63 expression not only in basal cells, but throughout the suprabasal layer of epithelial cells, in the dermis and around dermal blood vessels (Fig. 5A and C). No signal was detected when primary antibody was omitted during the staining procedure (Fig. 5B and D). The pattern of immunocytochemical staining suggests that this distribution reflects the high number of proliferating cells in keloids relative to normal skin, in which proliferation is restricted to the basal proliferating compartment of the epithelium.

Fig. 2 Real-time RT-PCR analysis of the mRNA for the ΔN-p63 isoform. Primers specific for the ΔN-p63 mRNA (1, 2), or the TA-p63 mRNA (3, 4), were used to screen RNA from normal skin (1, 3) and keloid (2, 4) specimens. Each RNA was quantified in a separate reaction, relative to the β-actin cDNA (= 100) as an internal cDNA control. Each *bar* represents the ratio of target PCR product to the β-actin control, and each value is the mean of three independent experiments
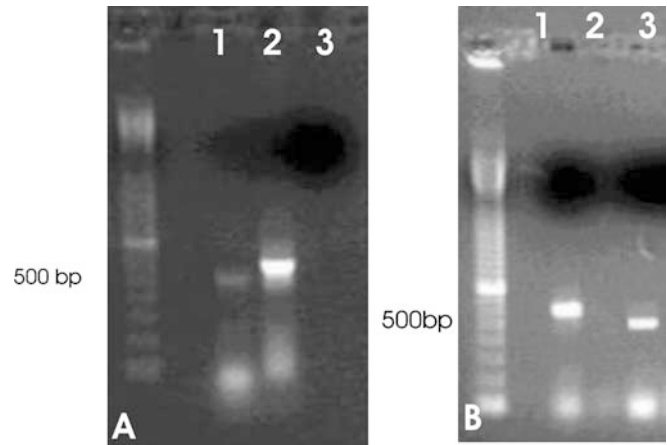
Discussion

We have identified a cDNA for the ΔN-p63 gamma isotype of p63, which is expressed specifically in human keloids. p63 is a member of the family which includes the structurally related proteins p53 and p73.

The identification of two homologues, p63 and p73, revealed that the tumor suppressor p53 is a member of a family of related transcription factors. Since they share amino acid sequence identity of 63% in the DNA-binding domain, p53, p63, and p73 may have redundant functions in the regulation of gene expression. Furthermore, one specific class of p63 proteins—those that lack an N-terminal transactivation domain—can strongly inhibit p53 and p63 activity (Yang et al. 1988).

In contrast to the case with p53, somatic mutations in the p63 gene are very rare. It appears that alterations in
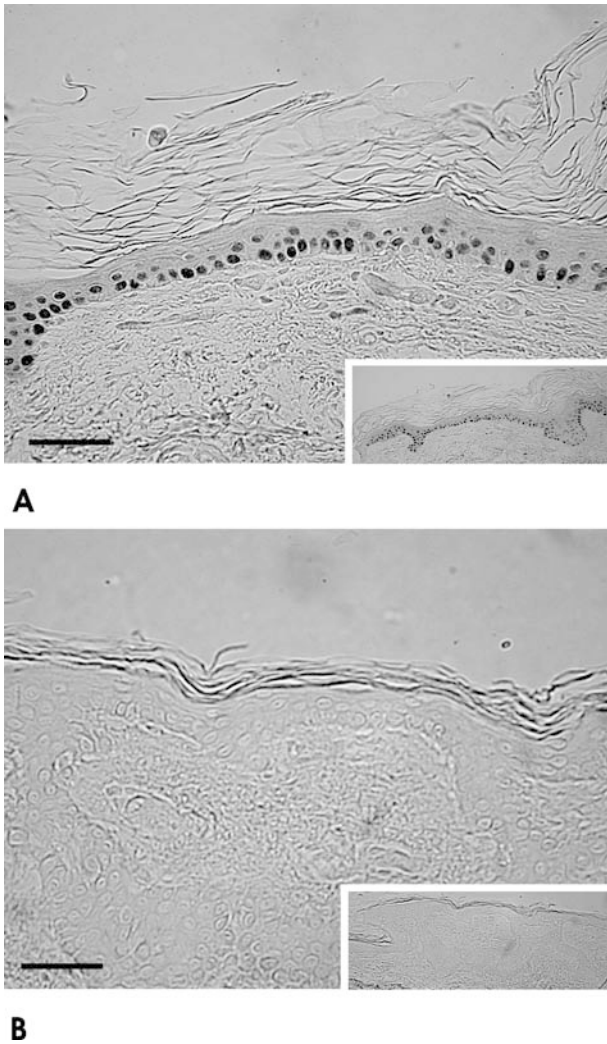
Fig. 4A, B Immunostaining of p63 in normal skin specimens with a monoclonal antibody directed against p63, detected using an alkaline phosphatase reporter system. **A** Positive cell nuclei (*brown*) are found throughout the basal layer of epithelial cells. In panel **B** the primary antibody was omitted during the staining procedure. *Bar* = 50 µm

p63 expression are pathological in nature. Our data confirm earlier results which showed that p63 is expressed in a restricted pattern in normal tissues (Fig. 4).

It has already been reported that keloids are positive for p53, but that keloid-derived fibroblasts show a lower rate of apoptosis than normal fibroblasts (Ladin et al. 1998).

These data support our observation of weak p53 expression in keloids (Fig. 3), while p63 is present at high levels in the nuclei of scattered cells in all epithelial layers and in the dermis in keloid tissues (Fig. 5).

The discovery that ΔN-p63 is the dominant isotype of p63 in keloids (Fig. 2) suggests a possible mechanism by which p53 is prevented from functioning as a transactivator in these tissues. The overexpression of the truncated isoform of p63 protein in all epithelial cell layers is significant. In fact, the cells of the basal layer of the

epithelium may act as progenitors of the suprabasal cells, which undergo differentiation and cell death. In accordance with this idea, in normal epithelial tissues, p63 is found only in basal cells that have a role in the regenerative processes in these epithelia (Yang and McKeon 2000).

Recent data from p63 knockout mice demonstrate that p63 is a key regulator of proliferation and differentiation programs which act to maintain the regenerative or "immortal" quality of epithelial stem cells (Mills et al. 1999; Yang and McKeon 2000). Indeed, p63$^{-/-}$ mice have no hair follicles, no teeth, no mammary, lachrymal or salivary glands (Yang et al. 1999). Data from other studies on p63 overexpression in tumor cells support an important role for p63 in the development of human primary tumors, including squamous and transitional cell carcinomas (Nishi et al. 1999; Park et al. 2000; Yamaguchi et al. 2000).

Our observation of increased expression of ΔNp63 in keloids is consistent with the aberrant growth seen in these tissues and suggests that aberrant p63 expression may underlie a neoplastic and proliferative potential in vivo, even in cases of benign hyperproliferation.

It has also been reported that p63 overexpression can mimic p53 activities by binding DNA, activating transcription, and inducing apoptosis (Kaelin 1999). However, unlike transcripts of the p53 gene, mRNAs for both p63 and p73 can undergo alternative splicing. For p63 three C terminal variants (α, β, and γ), two N terminal variants (TA and ΔN), and one interstitial splice variant in which 12 nt (encoding four amino acids) are deleted from exon 9, have been reported, leading to the formation of six different isoforms (Kaelin 1999). These isoforms may (TA forms) or may not (ΔN forms) contain the transactivation domain (TAD), depending on whether transcription of the precursor mRNA starts from exon I (TA forms) or exon III' (ΔN forms) (Levrero et al. 2000). Nothing is yet known about the factors that determine the choice between the two promoters. The HMG1-like protein SSRP1 has been found to stimulate p63 activity by associating with the intact form of p63γ at the promoter, but did not affect the residual activity of ΔN-p63γ (Zeng et al. 2002).

The three N-terminally truncated isoforms of p63 are unable to transactivate and to induce apoptosis. Furthermore, the same truncated isoforms appear to act in a dominant-negative manner to inhibit the transactivation potential of wild-type p53 and p63 (Yang et al. 1998). Our observation that the ΔN form of p63, which is devoid of the transcriptional activation domain, is the most highly expressed isoform in keloids suggests that this may represent an endogenous dominant negative regulator of p53 activity, an endogenous "anti-p53 protein."

In this context, we may speculate that when certain p63 isoforms, such as the dominant-negative ΔNp63, are overexpressed, they may bind to and inhibit transactivation by p53 and TAp63. Alternatively, this ΔNp63 isoform, by binding to specific promoter elements, could
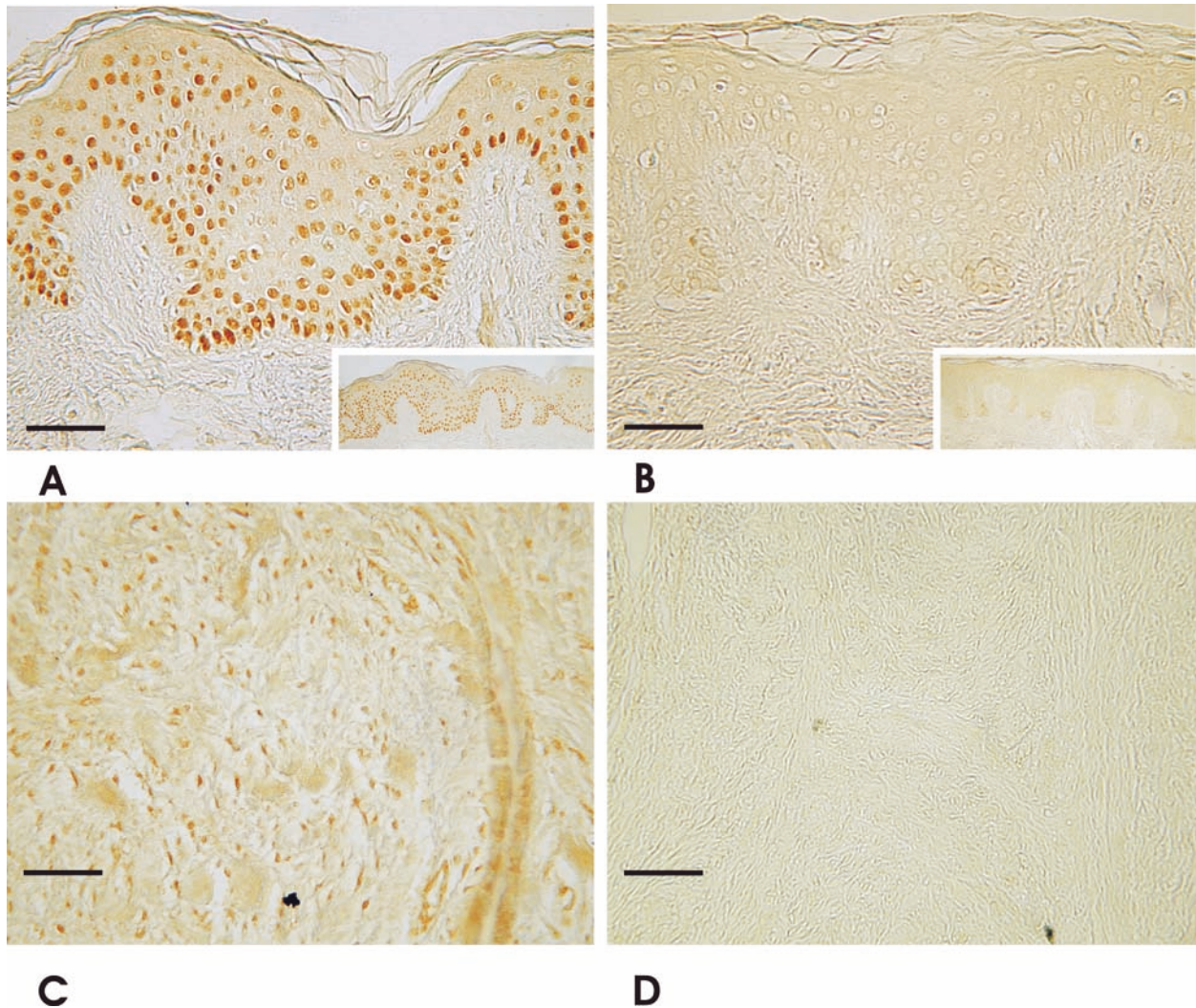
Fig. 5A–D Immunostaining of p63 in active keloid tissues with a monoclonal antibody directed against p63, detected using an alkaline phosphatase reporter system. **A** Positive cell nuclei (*brown*) are found throughout both the basal and suprabasal layers of epithelial cells. In panel **B** the primary antibody was omitted during the staining procedure. **C** Positive cell nuclei (*brown*) are also found in scattered fibroblast-like cells in the upper and middle dermis and around dermal blood vessels. In panel **D** the primary antibody was omitted during the staining procedure. *Bar* = 50 μm

block the transcription of otherwise critical genes, such as those involved in the apoptotic response and terminal differentiation. The observation that ΔNp63 is highly expressed in a hyperplasic tissue such as a keloid is at least compatible with such a hypothesis.

We therefore speculate that this isoform suppresses wild-type p53 functions and thus inhibits the expression of its downstream targets, which are required for processes including apoptosis, transient growth arrest, and sustained growth arrest or senescence. Further studies are necessary to elucidate the exact molecular mechanisms of p63/p53 expression and interaction, not only in malignant tumors, but also in benign tumors. But we nevertheless suggest that p63 could find application as a specific molecular marker for keloid formation.

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