Regulation of Cyclooxygenase-2 by Interferon γ and Transforming Growth Factor α in Normal Human Epidermal Keratinocytes and Squamous Carcinoma Cells

ROLE OF MITOGEN-ACTIVATED PROTEIN KINASES*

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The epidermis functions as a barrier to transepidermal water loss and defense against physical damage, microbes, UV light and xenobiotics (1–5). Differentiation in the epidermis begins with migration of basal cells into the spinous layer, followed by transit of the cells into the granular layer and subsequently into the stratum corneum (2, 5–7). Each of these stages is associated with induction of specific differentiation markers, including various keratins, transglutaminases, cornfin, and loricrin (4, 5, 7–11). Homeostasis in the epidermis is maintained by a balance between cellular proliferation, differentiation, and apoptosis (2, 5, 12). A variety of different signals, including several hormones and many cytokines, have been identified that influence these biological processes through autocrine, paracrine, or endocrine mechanisms (13–19). Dysregulation of the cytokine network has been implicated in many cutaneous diseases, including cancer and several inflammatory processes (14, 15, 20, 21).

Interferon-γ (IFN-γ) is a proinflammatory cytokine that is principally produced by activated T-lymphocytes and natural killer cells and affects a vast array of different cellular processes. IFN-γ has also been reported to affect growth and differentiation in cultured epidermal keratinocytes (14, 15, 22–25) and has been implicated in several inflammatory skin diseases, such as allergic contact dermatitis and psoriasis (14, 26–29). Sites of inflammation contain elevated levels of IFN-γ and interleukin-1 (ICAM-1), which plays a pivotal role in the adhesion and migration of leukocytes at sites of inflammation, and ICAM-1 is dramatically induced by IFN-γ in epidermal keratinocytes (26). Recent studies showed that targeted expression of IFN-γ to the suprabasal layers of the epidermis of transgenic mice induces increased proliferation, a thickened epidermis, perturbed differentiation, and eczema resembling contact dermatitis (27). These results demonstrate the importance of IFN-γ in the regulation of inflammation and cellular proliferation and differentiation in the skin.

Prostaglandins also play a major role in the induction of inflammatory processes in the epidermis and in the control of proliferation and differentiation of keratinocytes (30–34). Cyclooxygenases (COX-1 and COX-2) catalyze the first, rate-lim-
itening step in the conversion of arachidonic acid into prostaglandins and thromboxanes. COX-1 is constitutively expressed in a wide variety of tissues, including the epidermis, while COX-2 is a highly inducible gene that is expressed in response to a variety of proinflammatory agents and cytokines (35–43). The tumor promoter 12-tetradecanoylphorbol-13-myristate, epidermal growth factor, and UV irradiation have been shown to induce COX-2 in epidermal keratinocytes and in the epidermis (44–46). In addition to sites of inflammation, elevated COX-2 expression has also been found in many tumors, including skin (47–49). The COX-inhibitor indomethacin has been reported to suppress tumor formation in the skin (50), and COX-2 null mice developed 75% fewer chemically induced skin papillomas than control mice (51). These observations suggest that COX-2 has an important role in inflammation and carcinogenesis in the epidermis.

Although many inflammatory skin diseases are associated with increased levels of IFN-γ and prostaglandin E2 (PGE2), the relationship between IFN-γ, prostaglandin synthesis, and inflammation in the epidermis is not well understood. In this study, we demonstrate that IFN-γ induces COX-2 expression and increases PGE2 production in normal human epidermal keratinocyte (NHEK) cells. We provide evidence indicating that this induction is mediated at least in part through activation of the epidermal growth factor receptor (EGFR; c-ErbB1) and is related to increased expression of growth factors such as TGFα. This induction of COX-2 is regulated in part at the transcriptional level and involves the CRE/ATF site in the proximal COX-2 promoter region. In addition, we demonstrate the importance of the activation of both the ERK and p38 MAPK signaling pathways in COX-2 induction. The stimulation of TGFα synthesis and possibly other cytokines by IFN-γ and the subsequent increase in PGE2 production are likely to be important signals involved in triggering the hyperproliferative transformation associated with many inflammatory diseases in the skin.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Second passage cultures of NHEK isolated from human foreskin were obtained from Clonetics Corp. (San Diego, CA) and grown in keratinocyte growth medium-2 (KGM-2; Clonetics). The immortalized, nontransformed human epidermal keratinocyte cell line HaCaT, obtained from Dr. N. E. Fusenig (German Cancer Center, Heidelberg, Germany), and HPV-18-1 were described previously (52). The human squamous cell carcinoma cell lines SCC13 and SQCCY1 were obtained from Dr. J. G. Rheinwald (Harvard University, Boston, MA) and Dr. J. McLean (Hoffmann-La Roche, Nutley, NJ), respectively. All cell lines were maintained in KGM-2. Human recombinant IFN-γ, TGFα, and EGFR were purchased from R & D (Minneapolis, MN). In certain experiments, cells were treated with the EGFR kinase-selective inhibitor tyrphostin AG1478 or PD153035, the kinase inhibitor herbimycin A or genistein, the MEK inhibitor PD98059, or the p38 MAPK inhibitor PD169316 (Calbiochem).

**cDNA Probes—**Human cDNA probes for COX-1 and COX-2 were purchased from Oxford Biomedical Research (Oxford, MI). The plasmids pGAD-28 and pTG-7 encoding chicken glyceraldehyde-3-dehydrogenase (GAPDH) and rabbit transglutaminase type I, respectively, were described previously (10). Plasmids encoding rat TGFα and amphiregulin were kindly provided by Dr. D. Lee (University of North Carolina, Chapel Hill, NC) and Dr. M. Pettiolkow (Mayo Clinic/Foundation, Rochester, MN), respectively. The cDNA for 15(S)-lipoxigenase-2 was generated by RT-PCR as described previously (54). All probes were gel-purified and labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) via random priming using a kit and protocols supplied by Stratagene (La Jolla, CA).—Total RNA from cultured cells was isolated using Tri-Reagent (Sigma) according to the manufacturer's protocol. RNA (30 μg) was electrophoresed through a 1.2% denaturing agarose-formaldehyde gel and transferred to Nytran-plus membrane (Schleicher & Schuell) and then cross-linked by UV irradiation. Northern blots were prehybridized and hybridized in QuikHyb reagent (Stratagene) according to the manufacturer's protocol. Blots were washed at a final stringency of 60 °C in 0.2%/SSC, 0.1% SDS and then exposed to Hyperfilm MP (Amersham Pharmacia Biotech) at −70 °C. All significant results were confirmed in two or more independent experiments.

**Western Blot Analysis—**Cells were washed in PBS and then collected in sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin, 1 mM Na3VO4, 1 mM NaF). Protein concentration was determined by densitometry of the DC protein assay (Bio-Rad). Proteins (20 μg) were examined by immunoblot analysis as described previously (55) using Immobilon-P membranes (Millipore Corp., Bedford MA) and anti-COX-1 and COX-2 antibodies purchased from Oxford Biomedical Research (Oxford, MI). Peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:20,000 dilution; Chemicon, Temecula, CA) was used as secondary antibody. Antibodies were diluted in PBS containing 1% or 5% milk powder and 0.05% Tween 20. Detection was carried out by chemiluminescence using the SuperSignal CL-HRP substrate system from Pierce.

**Analysis of Arachidonic Acid Metabolites—**NHEK cells (five 150-cm2 dishes each) were treated with or without IFN-γ and were washed twice with PBS and collected by scraping in 1 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 1 μg/ml leupeptin and pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride). Cells were homogenized using a French Press (1.0 mg of protein in 1 ml) was diluted 1:1 with reaction buffer (100 mM Tris-HCl, 10 mM CaCl2) and incubated with [14C]labeled arachidonic acid (3 μCi, 25 μM) (NEN Life Science Products) at 37 °C for 30 min. Eicosanoids were extracted from the incubation buffer by acidification to pH 3.5 with acetic acid and applied to a C18-PrepSep solid phase extraction column (Waters) pretreated with methanol. The samples were then washed with acidified water, eluted with methanol, evaporated to dryness, and reconstituted with HPLC solvent. The eicosanoids were then analyzed by reverse-phase HPLC using an Ultrasphere ODS column (5 mm; 4.6 × 250 mm; Beckman). The solvent system consisted of a methanol/water gradient at flow rate of 1.1 ml/min as described previously (56). Radioactivity was monitored using a Flow Scintillation Analyzer (Packard) with Ecolume (ICN Biochemicals) as the liquid scintillation mixture. UV analysis was performed by monitoring absorbance at 235 nm with a Waters 990 photodiaray detector. Authentic standards 15(S)-HETE, PGE2, and prostaglandin F2α, were obtained from Cayman Chemical. PGE2, Radioimmunoassay—NHEK cells (1.5 × 106) were plated in six-well culture dishes in KGM-2 medium. After 24 h, cells were washed twice with PBS and medium was replaced with KGM-2 minus hydrocortisone (KGM-2 minus). NHEK cells were then treated with or without IFN-γ (200 units/ml) or TGFα either in the presence or absence of the anti-EGFR antibody LA-1 (5 μg/ml; Upstate Biotechnology, Inc., Lake Placid, NY). After 32 h, cells were incubated for an additional 30 min in fresh medium containing 10 μM arachidonic acid. The medium was removed, centrifuged, and stored at −70 °C. PGE2 levels were determined by Northern blot analysis using radiolabeled probes for COX-2 and GAPDH. Hybridization signals were quantitated with a Silverscanner IV (LACIE, Beaverton, OR) using NIH Image software. The levels of COX-2 RNA were normalized for the intensity of the GAPDH signal, which did not alter significantly. In some experiments, cells were treated simultaneously with actinomycin D and MAPK inhibitors PD98059 (60 μM) or PD168316 (5 μM), or anti-EGFR antibody LA-1. A Nuclear Run-off—The human COX-2 cDNA for nuclear run-off was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). NHEK-HPV-18 (4 × 106 cells) was plated in 10 150-mm dishes and grown in KGM-2 until approximately 80% confluence. Cells were then treated with or without IFN-γ (200 units/ml) for 20 h. Nuclei were isolated according to the procedure described by Dignam et al. (57) and stored at −70 °C. For the transcription assay, nuclei (1 × 107) were then washed in incubation buffer (4 μl Tris-HCl pH 8, 5 mM MgCl2, and 0.3 M KCl) containing 100 μg of uridine 5′-O[(α-32P)]triphosphate and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The human COX-2 and 18 S rRNA cDNA were fixed onto nitrocellulose membrane and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42 °C for 24 h using equal cpm/ml of labeled nascent RNA. "2 B. L. Harvat, and A. M. Jetten, manuscript in preparation."
treated with IFN-γ (200 units/ml) for the time indicated. A, total RNA was isolated and examined by Northern blot analysis using radiolabeled probes for COX-1, COX-2, transglutaminase type 1 (TG-1), and GAPDH. The size of the mRNAs is indicated on the right; 30 μg of RNA was loaded per lane. B, hybridization signals were quantitated with a Silverscanner IV as described under "Experimental Procedures" and normalized for the intensity of the GAPDH signal. The relative RNA levels were then calculated and plotted as follows: COX-2 (●), COX-1 (○), transglutaminase I (△).

RESULTS

Induction of COX-2 Expression by IFN-γ in NHEK—Previous studies have demonstrated that IFN-γ induces growth arrest and expression of several squamous differentiation markers in cultured NHEK (15, 23). To examine the effects of IFN-γ on the expression of COX in NHEK, cells were treated with IFN-γ, and the levels of COX-1 and COX-2 protein and mRNA were analyzed by Western blot and Northern blot analysis, respectively. Undifferentiated, exponentially growing NHEK cells expressed low levels of COX-1 and COX-2 mRNA (Fig. 1A). Treatment of NHEK with IFN-γ caused a small, transient increase in COX-1 mRNA expression (Fig. 1), while the level of COX-1 protein was moderately reduced (Fig. 2). In contrast, IFN-γ treatment enhanced the expression of COX-2 mRNA about 8-fold (Fig. 1B). The level of the 4.5- and 2.7-kilobase COX-2 transcripts increased between 8 and 16 h and reached a maximum after 24 h. The two COX-2 transcripts present in NHEK may be derived by the use of alternative polyadenylation signals. The time course of induction of COX-2 mRNA was very similar to the increase in the expression of the squamous differentiation-specific gene transglutaminase I. The 8-h delay in the induction of the COX-2 and transglutaminase I may suggest that these genes are regulated by IFN-γ through an indirect mechanism. The induction of COX-2 mRNA expression by IFN-γ was accompanied by an increase in COX-2 protein. COX-2 protein was undetectable in undifferentiated, exponentially growing NHEK cells and was dramatically increased between 16 and 24 h of IFN-γ treatment (Fig. 2A), and levels stayed steady over the remaining period tested (up to 48 h). The induction of COX-2 was dose-dependent (Fig. 2B). The level of COX-2 protein was increased at IFN-γ concentrations as low as 3 units/ml and reached a maximum at 30 units/ml. These observations demonstrate that IFN-γ is a strong inducer of COX-2 expression in NHEK cells.

Increased Synthesis of PGE2 by IFN-γ in NHEK—To determine whether the increase in COX-2 expression by IFN-γ resulted in any changes in arachidonic acid metabolism, the ability of cellular homogenates prepared from IFN-γ-treated and untreated NHEK cells to metabolize 14C-labeled arachidonic acid was analyzed by HPLC. HPLC profiles showed PGE2 and 15(S)-HETE as the two major metabolites synthesized by both untreated and IFN-γ-treated NHEK cells (Fig. 3, A and B). Homogenates from IFN-γ-treated cells exhibited an increased ability to metabolize arachidonic acid to PGE2 (Figs. 3 and 4) compared with control NHEK cells, in agreement with...
the observed induction of COX-2 expression. HPLC analysis also indicated an increased ability of IFN-\(\gamma\)-treated NHEK homogenates to synthesize various HETEs, including 15(S)-HETE. The latter may at least in part be related to the observed 2-fold increase in the level of 15-LO-2 mRNA expression (not shown), a new 15(S)-lipooxygenase recently described in the epidermis (54). Increased levels of HETE metabolites have been found in several skin diseases, including psoriasis and dermatoses. Although the precise role of these metabolites in the pathophysiology of the skin has yet to be determined, our results suggest that the increased synthesis of PGE2 and 15(S)-HETE in NHEK cells by IFN-\(\gamma\) is at least in part related to increased COX-2 and 15-LO-2 expression.

Transcriptional Regulation of COX-2 by IFN-\(\gamma\)—To determine whether the induction of COX-2 by IFN-\(\gamma\) is controlled at a translational or/and transcriptional level, the effect of IFN-\(\gamma\) on the stability of COX-2 mRNA and the rate of transcription was investigated. To examine the effect of IFN-\(\gamma\) on COX-2 mRNA stability, untreated NHEK cells and NHEK cells treated for 18 h with IFN-\(\gamma\) were incubated in the presence of the RNA synthesis inhibitor 5,6-dichlorobenzimidazole, and at different time intervals RNA was isolated and subjected to Northern blot analysis. The relative level of COX-2 mRNA was calculated from the densitometric analysis and plotted (Fig. 5). These results showed that IFN-\(\gamma\) had little effect on the stability of COX-2 mRNA. A slight decrease in stability was observed when actinomycin D was used (Fig. 5). These results suggest that IFN-\(\gamma\) regulates COX-2 mRNA expression largely at the level of transcription. This conclusion was supported by nuclear run-off analysis. This assay showed that the relative rate of COX-2 transcription in cells treated for 20 h with IFN-\(\gamma\) was about 3–4-fold higher than that in control cells (Fig. 6).

Induction of COX-2 in NHEK by IFN-\(\gamma\) Involves EGFR Activation—As demonstrated in Fig. 1, the prolonged time required for the induction of COX-2 mRNA suggests that COX-2 expression is regulated by IFN-\(\gamma\) by an indirect mechanism. The simultaneous increases in the levels of IFN-\(\gamma\), TGF\(\alpha\), and COX-2 in inflammatory skin disease suggest that there may be a link between these events. A recent study has implicated TGF\(\alpha\) in the induction of COX-2 by IFN-\(\gamma\) in human tracheobronchial epithelial cells (42). To determine whether the induction of COX-2 in NHEK cells was mediated by increased expression of other cytokines, we examined the effect of IFN-\(\gamma\) on the expression of two members of the EGF family, TGF\(\alpha\) and amphiregulin. Fig. 7A shows that treatment of NHEK with IFN-\(\gamma\) greatly enhanced the expression of TGF\(\alpha\) mRNA, while the level of amphiregulin mRNA was slightly and transiently increased. We also demonstrated that TGF\(\alpha\) increased COX-2 and TGF\(\alpha\) mRNA expression in NHEK cells (Fig. 7B) in agreement with previous studies (19, 45). The autinduction of TGF\(\alpha\) is likely to result in a greater enhancement of COX-2 expression. The demonstration that TGF\(\alpha\) can induce COX-2 mRNA within 2 h of treatment (Fig. 7C) and the temporal correlation between TGF\(\alpha\) and COX-2 mRNA induction (compare Figs. 1 and 7A) are in agreement with the hypothesis that the induction of COX-2 by IFN-\(\gamma\) is mediated by TGF\(\alpha\). The induction of COX-2 mRNA by TGF\(\alpha\) was accompanied by increased levels of PGE\(_2\) (Fig. 4). These results suggest that the induction of
COX-2 by IFN-γ in NHEK cells is at least in part mediated by increased expression of TGFα and possibly other cytokines that bind and activate the EGFR signaling pathway. This interpretation was supported by observations showing that an antibody against the EGFR greatly inhibited the induction of COX-2 mRNA expression (Fig. 7B) and PGE2 production (Fig. 4) by IFN-γ. In addition, the EGFR-selective kinase inhibitors PD153035 and tyrphostin AG1478 totally blocked the induction of COX-2 mRNA by IFN-γ (Fig. 8A). Genistein and herbi-

**FIG. 4.** Increased production of PGE2 by IFN-γ and TGFα in NHEK cells. Cells were treated with TGFα (50 ng/ml) or IFN-γ (200 units/ml) or vehicle for 22 h in the presence or absence of the anti-EGFR antibody LA-1. Cells were washed and then incubated in the presence of 10 μM arachidonic acid for 30 min. In control NHEK cells, LA-1 reduced PGE2 by 40–50% (not shown). The level of PGE2 released into the medium was determined by a radioimmune assay as described under "Experimental Procedures." The experiment shown is one of three with similar results.

**FIG. 5.** IFN-γ has little effect on the stability of COX-2 mRNA. NHEK cells were treated with IFN-γ (200 units/ml; closed symbols) or vehicle (open symbols) for 48 h and then incubated in the presence of actinomycin D (squares) or 5,6-dichlorobenzimidazole (20 μg/ml; circles). At different time intervals, cells were collected, and RNA was isolated. RNA was examined by Northern blot analysis using radiolabeled probes for COX-2 and GAPDH. Hybridization signals were quantitated with a Silverscanner IV, and the relative level of COX-2 mRNA was calculated. COX-2 mRNA levels were normalized for the level of GAPDH mRNA, which did not alter significantly during the time course of the experiment. The data represent one of two separate experiments with similar results.

**FIG. 6.** The induction of COX-2 expression by IFN-γ is regulated at the transcriptional level. Nuclei were isolated from NHEK cells treated with (lane 2) and without (lane 1) IFN-γ (200 units/ml) for 20 h as described under "Experimental Procedures." The rate of tran-
scription of COX-2 and 18 S rRNA (control) was determined by nuclear run-off assays. Newly synthesized RNA was slot-blotted and analyzed with radiolabeled probes for COX-2 and 18 S rRNA. Densitometry was performed, and the rate of COX-2 transcription relative to that of 18 S rRNA was calculated. These ratios were 44 and 163 for untreated and treated cells, respectively.

**FIG. 7.** A, effect of IFN-γ on the expression of TGFα and amphiregulin mRNA in NHEK cells. Cells were treated with IFN-γ (200 units/ml), and at the times indicated total RNA was isolated and examined by Northern blot analysis using radiolabeled probes for TGFα, amphiregu-
lin, and GAPDH. B, anti-EGFR antibody LA-1 blocks COX induction by IFN-γ. Cells were treated with TGFα (50 ng/ml) or IFN-γ (200 units/ml) in the presence or absence of LA-1 for 24 h. RNA was examined by Northern blot analysis with probes for TGFα, COX-2, or GAPDH. NA, untreated NHEK cells. C, induction of COX-2 mRNA by TGFα occurs rapidly. NHEK cells were treated for 24 h with LA-1 antibody. Cells were then washed and incubated in new medium in the presence (+) or absence (−) of TGFα (50 ng/ml). After 2 h, cells were collected, and RNA was examined by Northern analysis with probes for GAPDH and COX-2.

**Regulation of COX-2 by IFN-γ and TGFα**

mycin A, two other tyrosine kinase inhibitors that inhibit EGFR phosphorylation were also able to block COX-2 induction by IFN-γ. These inhibitors are, however, much less specific and may also inhibit other steps in the IFN-γ or TGFα signaling pathway.

**Role of MAPKs in the Control of COX-2 Expression—Activat-
tion of MAP kinases has recently been implicated in the regulation of COX-2 (59, 60). Growth factors, such as EGF, and cytokines can alter gene expression through phosphorylation of transcription factors via the activation of MAP kinase signaling pathways (61). Both the MEK inhibitor PD98059 and the p38 MAP kinase inhibitor PD169316 blocked both the IFN-γ and TGFα-induced COX-2 mRNA expression (Fig. 8, B and C). PD169316 and PD98059 did not affect the induction of the STAT1 gene, a target gene for IFN-γ, indicating that these inhibitors do not affect early steps in the IFN-γ signaling pathway. These results suggest a role for both ERK and p38 signaling pathways in the regulation of COX-2 expression by IFN-γ and TGFα.

We next examined the effect of PD98059 and PD169316 on the stability of COX-2 mRNA in TGFα-treated NHEK cells. As shown in Fig. 9A, the p38 MAPK inhibitor PD169316 caused a dramatic decrease in the stability of COX-2 mRNA, whereas the MEK inhibitor PD98059 exhibited in two independent experiments little effect on COX-2 mRNA stability in comparison with untreated cells. These results suggest that the activation of p38 MAPK regulates the stability of COX-2 mRNA, while activation of the ERK signaling pathway appears important in the transcriptional control of COX-2.

Importance of CRE/ATF/E-box in the Transcriptional Regulation of COX-2 by TGFα—The regulation of COX-2 by TGFα was further analyzed by comparing the transcriptional activity of different promoter flanking regions of the COX-2 gene in NHEK cells treated with and without TGFα by transient transfection of the respective luciferase reporter constructs. The transcriptional activation of the reporter gene through the minimal 252 promoter was very low with little difference between TGFα-treated and -untreated NHEK cells. The 2124 bp reporter construct significantly increased promoter activity in both untreated and TGFα-treated cells; however, the promoter activity in TGFα-treated NHEK cells was about 4–6-fold higher than in untreated cells (Fig. 10). The promoter activity further increased with the 2220, 2327, and 21432 promoter flanking regions; however, the ratio of transactivating activity between TGFα-treated and -untreated NHEK cells remained very similar. These results support the concept that the regulation of COX-2 expression by TGFα occurs at the transcriptional level and suggest that the CRE/ATF/E-box element plays an important role in the transcriptional control of COX-2 by TGFα. This conclusion was strongly supported by our observations showing that mutations in the CRE/ATF/E-box element...
TTCGTCACGTG dramatically reduced the promoter activity in TGF-α-treated and -untreated NHEK cells by almost 90%. A mutation in the NF-κB element had little effect on promoter activity, suggesting that this site is not important in the regulation of COX-2 by TGF-α, while mutations in C/EBP (TTACGCAAT → TTggtaccT) inhibited promoter activity in both untreated and TGF-α-treated cells about 50%, indicating also a role for this site in COX-2 regulation in these cells (Fig. 10C).

Defective Regulation in Carcinoma Cells—Squamous carcinoma cells have been reported to exhibit many changes in the control of growth and differentiation, some of which involve alterations in growth factor and cytokine signaling pathways. Recently, we showed that squamous carcinoma cell lines are rather refractory to the growth-inhibitory actions of IFN-γ (15). Therefore, we examined the effect of IFN-γ on COX expression in two immortalized cells, NHEK-HPV-18 and HaCaT, and two squamous carcinoma cell lines, SQCC/Y1 and SCC13. Both NHEK-HPV-18 and SQCC/Y1 cells expressed relatively high levels of COX-1 mRNA that were reduced by IFN-γ. HaCaT expressed low levels of COX-1, which increased slightly after IFN-γ treatment, while COX-1 mRNA was undetectable in SCC13 cells (Fig. 11A). IFN-γ either did not induce or only slightly increased COX-2 mRNA in HaCaT, SQCC/Y1, and SCC13 cells. These cells were found to be resistant to the growth-inhibitory and differentiation-inducing effects of IFN-γ. A small induction of COX-2 mRNA could be observed in SCC13 cells, and COX-2 protein was detectable after longer exposure (not shown). In contrast, in the HPV-18 immortalized NHEK cells, which remain sensitive to IFN-γ-induced growth arrest and differentiation, IFN-γ induced COX-2 mRNA and protein expression.

**Fig. 10. Analysis of the promoter activity of the 5′-regulatory region of the human COX-2 gene.** The promoter activity of a series of 5′-deletion mutants made in the COX-2 promoter flanking region was analyzed by transient transfection into NHEK cells treated with and without TGF-α (50 ng/ml) as described under “Experimental Procedures.” A, 5′-regulatory region of the human COX-2 gene. The TATA-box and several enhancer sites are indicated. Deletion mutants of the COX-2 promoter constructs are named by the length of the regulatory region. B, the relative promoter activity of each region was calculated (mean ± S.E.) and plotted. The fold increase in promoter activity between TGF-α-treated and untreated NHEK cells is shown on the right. Similar results were obtained in two other independent experiments. C, effect of mutations (indicated by an asterisk) in the CRE/ATF, C/EBP, or NF-κB site on the promoter activity of the −327 bp regulatory region. A similar result was obtained in one other independent experiment.

**Fig. 11. Induction of COX-2 expression by IFN-γ in several immortalized and squamous carcinoma cell lines.** Logarithmic cultures of NHEK-HPV-18, HaCaT, SQCC/Y1, and SCC13 cells were treated with and without IFN-γ (250 units/ml) for 48 h. A, isolated total RNA (30 μg) was examined by Northern blot analysis for expression of COX-1 and COX-2 mRNA. B, cells were collected, and proteins were examined by Western blot analysis using antibodies specific for COX-1 and COX-2.

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protein similar to NHEK cells (Fig. 11, A and B). The ability of IFN-γ to induce COX-2 correlated well with its ability to induce TGFα. IFN-γ had little effect on TGFα expression in SQCC/Y1, SCC13, or HaCaT cells but enhanced TGFα expression in NHEK-HPV-18 (Fig. 11A). We next examined the response of these cell lines to TGFα. Both TGFα and EGF were able to induce TGFα and COX-2 mRNA expression in SCC13 and NHEK-HPV-18 cells (Fig. 12). However, HaCaT and SQCC/Y1 cells were rather refractory to TGFα. Although an increase in TGFα mRNA was observed (Fig. 12), the level remained much lower than that in SCC13 cells, while induction of COX-2 mRNA could be seen only after long exposure (not shown). These results suggest that HaCaT and SQCC/Y1 are refractory to the COX-2-inducing effects of both IFN-γ and TGFα, while SCC13 is refractory to IFN-γ but not TGFα. The level of COX-2 expression in SCC13 was very much dependent on cell density (not shown). In contrast to low density, confluent cultures expressed high levels of COX-2 mRNA; this variation is probably related to endogenous expression of TGFα and increased accumulation of autocrine factors, such as TGFα, in the medium at high density.

DISCUSSION

IFN-γ has been reported to regulate the expression of COX-2 in several cell systems. In human bronchial epithelial cells and macrophages (42, 62), IFN-γ induces COX-2, while it has no effect in osteoblast and smooth muscle cells (41) and inhibits COX-2 expression in microglial cells (63). In this study, we demonstrate that in NHEK IFN-γ, treatment causes a dramatic induction in the expression of COX-2, while COX-1 expression is inhibited. The increase in the level of COX-2 protein by IFN-γ is probably responsible for the observed increase in PGE2 synthesis. We show that the induction of COX-2 by IFN-γ in NHEK is at least in part mediated through activation of the EGFR signaling pathway due to observed increased expression of TGFα and possibly other growth factors by IFN-γ. A similar mechanism has recently been reported for human bronchial epithelial cells (42). The rapid increase in COX-2 expression by TGFα in NHEK (Fig. 7C) as well as other cell types (40, 42, 45, 64) and the temporal correlation between the induction of TGFα and COX are in agreement with such a mechanism. Observations showing that anti-EGFR antibodies and EGFR-selective kinase inhibitors almost totally block induction of COX-2 and PGE2 by IFN-γ suggest that activation of EGFR is a major signaling pathway involved in the up-regulation of COX-2 by IFN-γ. These findings are highly relevant to understanding the role that IFN-γ plays in inflammatory skin disease. Inflammatory processes in the skin, including psoriasis and dermatitis, are characterized by hyperplasia and the presence of high levels of IFN-γ as well as of TGFα and PGE2 (13, 13).
putative enhancer elements, including C/EBP, CRE/ATF, NF-

The COX-2 promoter has been shown to contain a number of

as well as posttranscriptional (38, 73, 74) mechanisms. COX-2 mRNA is relatively unstable, and its stability is thought to be controlled by the multiple copies of the AUUUA instability motif in its 3′-untranslated region (73, 75). In lung carcinoma A549 cells, the induction of COX-2 by IL-1β has been reported to occur at a posttranscriptional level (73), and the increase in COX-2 mRNA levels by IL-1α in human endothelial cells appears also related to increased RNA stability (38). In many systems, the induction of COX-2 has been demonstrated to be regulated at the transcriptional level. The high expression levels of COX-2 mRNA in colon and skin carcinomas and transformed mammary epithelial cells relative to normal cells (47, 70, 72) as well as the induction of COX-2 by several growth factors, phorbol esters, and interleukins in several cell types has been reported to be controlled at the transcriptional level (40, 43, 65–69). In this study, we show that IFN-γ has little effect on the stability of COX-2 mRNA in NHEK cells. Nuclear run-off assays indicated that the induction of COX-2 expression by IFN-γ is related to an increase in the rate of transcription.

The COX-2 promoter has been shown to contain a number of putative enhancer elements, including C/EBP, CRE/ATF, NF-κB, E-box, STAT3, and AP2 (43, 66, 67, 72, 75). The up-regulation of COX-2 by hypoxia mediated through a NF-κB site (76). The induction of COX-2 by phorbol esters and lipopolysaccharide in vascular endothelial cells occurs at the transcriptional level and involves regulation through the C/EBP and CRE/ATF sites in the proximal promoter region (43). The v-Src induction of COX-2 in Balb/c 3T3 cells (66) and the increase in COX-2 expression by phorbol esters in oral carcinoma cells (40) require the CRE/ATF site, while the transcriptional regulation of COX-2 in mouse skin carcinomas is dependent on the C/EBP and E-box sites (72). Our results obtained by transient transfection assays using several reporter constructs, in which the reporter is under the control of various lengths of the COX-2 regulatory region, support the conclusion that TGFα regulates COX-2 gene expression at least in part at the transcriptional level. Deletion analysis of the −1432 bp regulatory region indicated that the −124 bp proximal promoter region containing the CRE/ATF/E-box element plays an important role in the transcriptional control by TGFα. This was confirmed by observations showing that mutations in the CRE/ATF/E-box element dramatically reduced promoter activity.

Recently, it was reported that COX-2 expression can be regulated through different MAP kinase signaling pathways and that the particular signaling pathway involved is dependent on the type of inducer (59, 60, 69, 71, 74, 77). The induction of COX-2 by PDGF has been demonstrated to require activation of the ERK signaling pathway (69), while constitutively active MEKK1 has been shown to induce COX-2 expression by activating the SEK1/MKK4-p38 kinase pathway (59). EGF/TGFα has been reported to activate several signaling pathways including STAT and MAPKs (61, 78–80). The suppression of IFN-γ/TGFα-induced COX-2 expression by the p38 kinase inhibitor PD169316 and MEK inhibitor PD98059 is in agreement with the concept that both the p38 and ERK MAPK signaling pathways are important in the regulation of COX-2 by IFN-γ/TGFα. Because the MEK inhibitor blocks the induction of COX-2 by both IFN-γ and TGFα but has no effect on the stability of COX-2 mRNA, activation of ERK appears to control COX-2 expression at the transcriptional level. The latter may involve phosphorylation and activation of transcription factors interacting with the CRE/ATF/E-box site. Activation of p38 appears to play a role in the regulation of COX-2 mRNA stability rather than transcription since PD169316 decreases COX-2 mRNA stability, while activation of p38 only (in NHEK treated with MEK inhibitor) is not sufficient to increase COX-2 mRNA. However, a synergistic action between the two MAPK signaling pathways on COX-2 transcription cannot be ruled out. A role for p38 MAPK in the control of COX-2 mRNA stability was recently also reported for lipopolysaccharide-treated monocytes (74). Although PD169316 results in decreased stability of COX-2 mRNA, little difference in COX-2 mRNA stability was found between IFN-γ-treated and untreated NHEK cells. These two events are not mutually exclusive, since p38 may already be activated in untreated NHEK cells. The latter is supported by the observed reduction of COX-2 levels by PD169316 in untreated cells (Fig. 8B).

The effects of IFN-γ on skin and NHEK cells are complex, and both growth-stimulatory and growth-inhibitory effects have been reported (14, 15, 23, 25–27). In the case of phorbol esters (81), which can also elicit growth-stimulatory as well as growth-inhibitory responses in epidermal keratinocytes, these opposite responses have been attributed to different subpopulations of keratinocytes: stem cells with a high proliferative capacity and transient amplifying (TA) cells, which undergo a very limited number of divisions before differentiating (6, 7). Stem cells are induced to proliferate in response to phorbol esters, whereas TA cells undergo squamous differentiation. It is possible that these two different cell populations respond to IFN-γ in a similar manner. In vivo, IFN-γ elicits a proliferative response in epidermal keratinocytes, and hyperproliferation of keratinocytes during inflammation is associated with an increase in IFN-γ, TGFα, and COX-2, in agreement with a positive role for IFN-γ in the regulation of cell proliferation in the skin (19–21, 26, 29, 30, 82). The induction of TGFα by IFN-γ provides a molecular mechanism that could explain the IFN-γ-induced keratinocyte proliferation and inflammation in vivo. A schematic model illustrating the relationship between the induction of TGFα and COX-2 by IFN-γ and the putative roles the ERK and p38 MAPK signaling pathways is shown in Fig. 13. Phosphorylation and activation of specific transcription factors lead to increased transcription of COX-2 and production of PGE2. Both increases in the level of PGE2 and TGFα may contribute to the inflammatory process.

Changes in the regulation of growth and differentiation in immortalized keratinocytes and squamous carcinoma cell lines have been attributed at least in part to alterations in cytokine and growth factor signaling pathways. Previous studies showed that IFN-γ had little effect on the growth of HaCaT, SQCC/Y1, and SCC13 cells (15). In this study, we show that IFN-γ affected the expression of COX-2 and TGFα in some of these cells only to a small extent. The action of IFN-γ on gene expression is mediated through the IFN-γ receptor and the subsequent activation of the JAK/STAT signaling pathway (83). We reported previously that these cells contain the IFN-γ receptor and are still responsive to IFN-γ as indicated by the induction of several target genes, including STAT1, IRF1, and the guanylate binding protein (15). These results suggest that early steps in the IFN-γ signaling pathways are functionally normal and indicate that the resistance to TGFα/COX-2-inducing effects of IFN-γ are due to changes downstream in the IFN-γ signaling pathway. The inability of IFN-γ to increase COX-2 mRNA could be related to its inability to induce TGFα expression. This appears to be the case for SCC13 cells in which
IFN-γ did not induce COX-2 or TGFα, while treatment with TGFα was able to dramatically enhance COX-2 expression. In contrast, TGFα was unable to significantly increase COX-2 expression in SCCq/1 and HacaT, suggesting that these cells harbor defects also in the TGFα signaling pathway.

Recently, we have demonstrated that the expression of PPARγ is associated with squamous cell differentiation (84). PPARγ is expressed in the suprabasal layers of the epidermis, and PPARγ and, to a lesser degree, PPARα are induced in cultured epidermal keratinocytes after treatment with phorbol esters and IFN-γ. A number of arachidonic and linoleic acid metabolites have been reported to bind and activate members of the PPAR nuclear receptor subfamily (53). Prostaglandin J2 metabolites have been reported to bind PPARγ, COX-2, and lipoxygenases in papillomas and carcinomas, respectively. PPARγ controls COX-2 and lipoxygenases, the parallel induction of PPAR expression with that of cyclooxygenase and lipoxygenase opens the possibility that agents, such as IFN-γ and phorbol esters, can indirectly control the activation of the PPAR signaling pathway and gene expression by regulating the production of specific PPAR ligands. The association of increased expression of PPARs, COX-2, and lipoxygenases in papillomas and carcinomas may support a role for this concept in these pathological processes.

In summary, in this study we demonstrate that the induction of COX-2 in NHEK cells by IFN-γ is mediated at least in part through activation of the EGFR signaling pathway due to increased expression of growth factors such as TGFα (Fig. 13). We cannot rule out the possibility that IFN-γ controls COX-2 through activation of other additional signaling pathways that may act synergistically with the EGFR pathway. In addition, we demonstrate that this induction of COX-2 is regulated at the transcriptional level and involves the CRE/ATF site in the proximal COX-2 promoter region. We provide evidence showing that activation of both the p38 and ERK MAP kinase signaling pathways plays an important role in the control of COX-2 expression. The stimulation of both the p38 and ERK MAP kinase signaling pathways plays an important role in the control of COX-2 expression. This stimulation of TGFα and possibly other cytokines by IFN-γ and the subsequent increase in PGE2 production are probably important signals that trigger the hyperproliferative transformation associated with many inflammatory skin diseases.

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