PROSTAGLANDIN-H-SYNTHASE ISOZYME EXPRESSION IN NORMAL AND NEOPLASTIC HUMAN SKIN

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Expression of prostaglandin-H-synthase (PGHS) isozymes was analyzed in 50 biopsies of normal human skin and of pre-malignant and malignant skin lesions, by means of quantitative RT-PCR, immunoprecipitation and Western blotting, as well as immunohistochemistry. Normal skin constitutively expressed PGHS-1 in all cell layers of the epidermis, in endothelial cells of small blood vessels and in sweat-gland epithelium. PGHS-2 expression was very low and restricted to a few keratinocytes of the interfollicular and follicular epidermis. Steady-state concentrations of PGHS-1 and PGHS-2 mRNA were similar in normal skin and in basal-cell carcinomas, but PGHS-1 mRNA was reduced and PGHS-2 mRNA was elevated in actinic keratoses, squamous-cell carcinomas and keratoacanthomas. PGHS-1 protein was detected in all tumor biopsies, being occasionally increased in basal-cell carcinomas. High amounts of PGHS-2 protein were found in actinic keratoses, squamous-cell carcinomas and keratoacanthomas, but not in basal-cell carcinomas. Four malignant melanomas included in this study contained PGHS-1 but no PGHS-2 protein. Immunohistochemical analysis of the biopsies identified keratinocytes, in addition to cells of inflammatory infiltrates and of dendritic morphology, as the major PGHS-expressing cell types. PGHS-2-specific signals were spread throughout the epidermal part of actinic keratoses and squamous-cell carcinomas. These data suggest that constitutive up-regulation of PGHS-2 expression is a consistent pre-malignant event in squamous-cell cancer development in man, as it is in animal models of skin carcinogenesis. Thus, pre-cancerous lesions such as actinic keratoses present a likely target for chemoprevention of skin cancer by selective PGHS-2 inhibitors. Int. J. Cancer 82:648-656, 1999.

A large body of evidence has now been accumulated from epidemiological studies (Smalley and DuBois, 1997) and from clinical studies, animal models and cell-culture experiments (Levy, 1997; DuBois *et al.*, 1998) to show that the regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) is a promising option for chemoprevention of colorectal cancer. The best-defined molecular targets of NSAIDs are the prostaglandin H synthases (PGHS; also termed cyclooxygenases, COX). Two PGHS isozymes are known that are encoded by individual genes and regulated differentially (Vane *et al.*, 1998). The isozyme PGHS-1 is constitutively expressed in most tissues and cells so far investigated, whereas the PGHS-2 isozyme is inducible in virtually all tissues, being the product of a pro-inflammatory-response gene that is readily activated upon induction of inflammatory and regenerative processes (DuBois *et al.*, 1998).

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Over-production of prostaglandins is a consistent property of many experimentally induced epithelial tumors (Müller-Decker *et al.*, 1995; Rao *et al.*, 1995). Studies on experimental models of intestinal carcinogenesis, such as azoxymethane-induced colon cancer in rats (Singh *et al.*, 1997) and spontaneous tumorigenesis in Min mice, which are genetically predisposed to intestinal carcinogenesis (Smalley and DuBois, 1997), revealed a correlation between tumor development and up-regulation of PGHS-2 expression in tumor cells. Moreover, pharmacological approaches showed that PGHS-2-selective inhibitors such as Celecoxib [also termed SC58635 (Kawamori *et al.*, 1998)] or MF-tricyclic (Oshima *et al.*,

1996) efficiently suppress tumor development in these model systems. More direct evidence for a causal relationship between PGHS-2 induction and colon-tumor formation was provided by experiments with mice genetically predisposed to intestinal tumorigenesis. In these animals a knock-out of the *PGHS-2* gene resulted in a marked reduction of tumor formation indicating that PGHS-2 induction is a rate-limiting step in intestinal-tumor formation (Oshima *et al.*, 1996).

Concerning the human situation, clearcut evidence for a chemopreventive effect of NSAIDs exists as yet only for colorectal cancer. In contrast, such an effect has been established for a variety of experimental animal tumors, including those of the epidermis, esophagus, stomach, colon, urinary bladder, kidney, lung and mammary gland (for review, see Marnett, 1992). Studies on multistage mouse-skin carcinogenesis (Fürstenberger et al., 1989) have provided important insights into the underlying mechanisms. Thus, PGHS-2 formation was specifically stimulated by treatment with phorbol-ester tumor promoters, while NSAIDs such as the non-selective PGHS inhibitor indomethacin and the selective PGHS-2 inhibitor SC58125 (Frölich, 1997) were found to be potent inhibitors of skin-tumor promotion (Fischer et al., 1987; Müller-Decker et al., 1998a). Moreover, this animal model showed the anti-tumor effect of NSAIDs to be related to prostaglandin formation, since the inhibitory activity of indomethacin could be reversed specifically by prostaglandin $F_{2\alpha}$ (Fürstenberger et al., 1989). In addition, the time course of PGHS-2 induction correlated with that of $PGF_{2\alpha}$ formation during tumor promotion. Constitutive over-expression of PGHS-2 in the proliferative-cell compartment was a consistent feature of the mouse-skin tumors thus obtained (Müller-Decker et al., 1998a).

When extrapolated to the human situation, these results would indicate that non-melanoma skin cancer in humans might be a target of chemoprevention by NSAIDs. Non-melanoma skin cancer is one of the most frequent neoplastic diseases, induced mainly by exogenous factors such as chemicals and, particularly, sunlight, and is thought to develop from defined pre-cancerous lesions (Byrd *et al.*, 1996; Brash, 1997). As precursor lesions for squamous-cell carcinomas, actinic keratoses may be potential targets for preventive measures.

In the light of results from animal studies and investigations into the mechanism of colorectal cancer, over-expression of prostaglandin synthases is considered an indicator for potential chemoprevention of human skin cancer by NSAIDs. For this reason, we analyzed the expression of PGHS isozymes in biopsies from normal human skin, neoplastic precursor lesions, and skin cancer.

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Here we show that up-regulation of PGHS-2 is a consistent parameter of non-melanoma human skin tumors.

MATERIAL AND METHODS

Material

Goat anti-human PGHS-1- (SC1754) and PGHS-2- (SC1745) anti-sera, horseradish-peroxidase-conjugated donkey anti-goat IgG and alkaline-phosphatase-conjugated donkey anti-goat IgG antibodies were obtained from Santa Cruz (Heidelberg, Germany). Alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody was purchased from Dianova (Hamburg, Germany).

Methods

Biopsies. After informed consent, skin biopsies were obtained from patients of the Departments of Dermatology at the University Hospitals of Rostock and Mannheim, Germany. For anesthesia, 1% Scandicain (Astra, Wedel, Germany) was used without any vasoconstrictor. First, the histologic diagnosis was evaluated by dermatopathologists using hematoxylin-eosin-stained cryostat or paraffin sections according to standard diagnostic criteria. Immediately after surgery, tissue samples were immersed in Tissue-Tek (Vogel Giessen, Germany), snap-frozen in liquid nitrogen, and stored at $-70^{\circ}\mathrm{C}$.

Homogenization of tissue samples. Frozen skin biopsies were ground by a pestle in a pre-cooled steel mortar and the ground tissue was pulverized in a ball mill (Retsch, Haan, Germany) for 10 sec. All steps were performed at the temperature of liquid nitrogen.

RNA isolation. Aliquots of the pulverized tissue samples were homogenized directly in guanidinium-isothiocyanate solution, the lysis buffer of the RNAClean extraction kit (AGS, Heidelberg, Germany), and total RNA was extracted according to the manufacturer's instructions.

Preparation of internal standards. A PGHS-1-specific cDNA fragment of 304 bp was amplified by polymerase chain reaction (PCR) using cDNA prepared from a human squamous-cell carcinoma with the forward primer 5'-TGCCCAGCTCCTGGCCCGC-CGCTT-3' (Nucleotide 521-544, h PGHS-1) and the reverse primer 5'-GTGCATCAACACAGGCGCCTCTTC-3' (Nucleotide 824-801). Similarly, a PGHS-2-specific cDNA fragment of 184 bp was obtained with the primers 5'-GACAGATGATAAGC-GAGGGCCAGC-3' (Nucleotide 688-711, h PGHS-2) and 5'-CTCTGCCTGAGTATCTTTGACTGT-3' (Nucleotide 871-848). The PCR products were cloned into the PCRII-vector (InVitrogen, Leek, The Netherlands), then subcloned into the pBluescript vector (Stratagene, Amsterdam, The Netherlands). An unrelated 144-bp fragment was inserted in an unique NcoI restriction site of the PGHS-1 insert to give plasmid pCOX-1 and a 72-bp fragment in the NcoI site of the *PGHS-2* insert to give plasmid pCOX-2. The inserts were sequenced by fluorescence-tagged dye terminator cycle sequencing (Amersham, Braunschweig, Germany) and analyzed on an ABI prism 310 Genetic analyzer (Perkin Elmer Applied Biosystems, Weiterstadt, Germany).

cRNAs were prepared from the linearized pCOX-1 and pCOX-2 template DNAs using T7 RNA polymerase. Template DNAs were digested with RNase-free DNAseI (Boehringer, Mannheim, Germany) and the cRNAs were purified according to the RNA transcription protocol of Strategene. Quantitation of the standard cRNA was determined by absorbance at 260 nm.

 $\it RT\text{-}PCR$. First, 100 pg PGHS-1 standard cRNA or 10 pg PGHS-2 standard cRNA were added to 5 μg total RNA, and first-strand cDNA synthesis was performed using the cDNA cycle kit (InVitrogen) and oligo dT primers according to the manufacturer's protocol.

PCR was primed with aliquots of 40%, 20% and 4% of the reverse-transcription reaction as templates using 100 pmol of primers, 10 mM Tris, pH 9.0, 50 mM KCl, 1.5 mM MgCl $_2$, 0.1% Triton X-100, 0.2 mM gelatin, 0.2 mM of each dNTP and 5 units

Taq DNA polymerase (Appligene-Oncor, Heidelberg, Germany) in a 100-µl reaction. Blank reactions with no template were carried out as negative control. The PCR was programmed in a PTC-200 DNA Engine (MJ Research, Biozym, Oldendorf, Germany) as follows: 94°C for 5 min, 1 cycle; 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, 30 cycles; 72°C for 10 min, 1 cycle; the block temperature was held at 4°C. For amplification of PGHS-2 amplicons, the annealing temperature was set to 58°C. With the above-mentioned PGHS-specific primers, the amplicon size was 448 bp for the internal PGHS-1 standard and 304 bp for the target PGHS-1 mRNA, while for the internal PGHS-2 standard it was 256 bp and for the target PGHS-2 mRNA 184 bp. Aliquots (10 µl) of the PCR reactions were electrophoresed in 1.4% agarose gels, visualized by ethidium-bromide staining, and photographed. Photographs were used to evaluate band density (Sigma Gel, Jandel, Meckenheim, Germany). The ratio of target PCR product to homologous internal standard PCR product was used to estimate the level of gene expression.

Protein determination. Protein concentrations were determined by means of the BioRad DC Protein Assay (BioRad, Munich, Germany), with BSA as standard.

Immunoprecipitation. Tissue powder was homogenized in buffer containing 50 mM Tris/HCl, pH 7.4, 1 mM diethyldithiocarbamate, 2 mM EDTA, 1.0% Tween 20, 0.2 mg/ml α_2 -macroglobulin, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin, 10 μ g/ml each. After centrifugation (4000 g, 4°C, 20 min), 1 to 1.5 mg of the supernatant protein was used for immunoprecipitation of PGHS-2 according to the procedure described (Müller-Decker et al., 1998b), PGHS-1 then being immunoprecipitated from the supernatant of the PGHS-2 precipitation.

Western-blot analysis. Immunoprecipitated proteins were electrophoresed using 7.5% SDS polyacrylamide gels. The proteins were electroblotted onto PVDF membranes as described (Scholz *et al.*, 1995). Thereafter, the membranes were handled according to the protocol recommended by the supplier of the TROPIX-Westernlight-chemiluminescence detection system (Perkin Elmer Applied Biosystems). Goat anti-human PGHS-2 (SC 1745) and rabbit anti-PGHS-1 antibodies (Müller-Decker *et al.*, 1998*b*) were used at a dilution of 1:2000 and the anti-goat/anti-rabbit IgG alkaline-phosphatase antibodies at a dilution of 1:2000/5000. Protein sizes were estimated by comparison with co-blotted molecular-weight-standard proteins (SDS-6H, Sigma, Deisenhofen, Germany).

Immunohistochemistry. Tissue samples were immersed in Tissue-Tek (Vogel), snap-frozen in liquid nitrogen, and stored at -70° C. Cryostat sections (5 µm) were fixed in 1% paraformaldehyde in PBS (5 min) and in absolute ethanol (5 min). Staining of specimens followed the protocol described (Müller-Decker et al., 1998b). Briefly, after blocking of endogenous peroxidase by incubation with 3% H₂O₂ in methanol for 10 min, specimens were blocked (2.5% skim milk powder in PBS, Fluka, Neu-Ulm, Germany) for 1 hr and incubated with goat anti-human PGHS-2 or goat antihuman PGHS-1 anti-serum (SC1745 or SC1754, respectively, diluted 1:25 in block solution for 16 hr at 4°C). The peroxidaseconjugated secondary antibody (donkey anti-goat-IgG horseradish peroxidase) diluted 1:200 in block solution was added for 1 hr at room temperature. Incubation with substrate (0.07% DAB, 0.16% hydrogen peroxide, Fast DAB tablets, Sigma, Munich, Germany) was for 1 hr at room temperature. Tissues counterstained with hematoxylin were mounted in eukitt. For assessment of the specificity of the immunoreaction, control sections from each tissue were incubated with primary antibodies adsorbed with the respective peptide antigen (Santa Cruz; 500-fold molar excess). As additional controls, unspecific binding of the secondary antibody or DAB to skin structures was checked by omitting the primary and secondary antibodies respectively. The sections were examined for staining using a Leitz orthoplan microscope. Photographs were taken on Fuji Color Super Gold films, 400 ASA, at 0.01 sec exposure.

RESULTS

In this study, we analyzed a total of 50 skin biopsies derived from different body sites, comprising 11 biopsies from normal skin, 11 actinic keratoses (AK), 1 carcinoma in situ (CIS), 8 squamouscell carcinomas (SCC), 2 keratoacanthomas (KA), 13 basal-cell carcinomas (BCC) and 4 malignant melanomas. The biopsies are listed in Table I, together with the body sites of origin. The mean age of patients was 68.5 ± 19.6 years; 36% of samples were from men and 64% from women.

The mRNA steady-state concentrations of PGHS-1 and PGHS-2 were determined by semi-quantitative RT-PCR. Internal standard cRNAs for PGHS-1 and PGHS-2 were synthesized from plasmids containing the target cDNAs with insertions of unrelated sequences of 144 and 72 bp respectively. cDNA synthesis was initiated with 5 μg of total RNA and cRNA standard templates, and PCR was performed on different dilutions of the cDNA reaction mixtures. The amplification products of the internal standards (484-bp band for PGHS-1 and 256-bp band for PGHS-2) and target genes (bands of 304 and 184 bp respectively) were analyzed by gel electrophoresis (Fig. 1), and ethidium-bromide staining was used for semiquantitative evaluation (Table II). As compared with normal skin, PGHS-1 mRNA contents were found to be reduced in the majority of the actinic keratoses, squamous-cell carcinomas and basal-cell carcinomas. The contents of PGHS-2 mRNA were very low in normal skin and in basal-cell carcinomas, but strongly increased in 3 out of 4 actinic keratoses and in one out of 2 squamous-cell carcinomas (Table II). Thus, with the exception of basal-cell carcinomas, a decrease of PGHS-1 mRNA and a simultaneous increase of PGHS-2 mRNA were a consistent feature of neoplastic as compared with normal skin.

Protein expression of PGHS isozymes was investigated using isozyme-selective immunoprecipitation and subsequent Westernblot analysis. A representative result is shown in Figure 2. It can be seen that PGHS-1-specific signals were delivered by all normal skin specimens (sample numbers 1, 7 and 10) and the tumor biopsies (24, 28, 29, 33, 35, 36, 43, 44, and 47). PGHS-2-specific signals were restricted to samples from neoplastic epidermis and exhibited pronounced inter-individual variations. Thus, very strong signals were found for the squamous-cell carcinomas 24 and 29 and the keratoacanthoma 33, whereas the squamous-cell carcinoma 28 showed a faint PGHS-2 signal, which became visible only after prolonged exposure of the film (Fig. 2). Of the basal-cell carcinomas (35, 36, 43, and 44), only the samples 35 and 36 were slightly PGHS-2-positive. No PGHS-2 protein could be detected in the malignant melanoma biopsy (sample 47, Fig. 2). Semi-quantitative evaluation of the PGHS-1 and -2 contents in all biopsies investigated so far is given in Table III. Actinic keratoses, the carcinoma

TABLE I - COLLECTION OF BIOPSIES1

Histology	Body site	Biopsy number	
Normal skin	Head	1–6	
	Thorax	7–9	
	Extremities	10, 11	
Actinic keratosis	Head	12–16	
	Thorax	17	
	Extremities	18-22	
Carcinoma in situ	Thorax	23	
Squamous-cell carcinoma	Unknown site	24-27	
-	Head	28-30	
	Extremities	31	
Keratoacanthoma	Head	32	
	Extremities	33	
Basal-cell carcinoma	Head	34-42	
	Thorax	43, 44	
	Extremities	45, 46	
Malignant melanoma	Extremities	47–50	

¹Information for each patient in this study includes histologic diagnosis and body site of biopsies.

in situ, squamous-cell carcinomas and keratoacanthomas were found to express PGHS-1 protein at a level comparable with that in normal skin. A more pronounced PGHS-1-specific signal was seen in 2 out of 8 basal-cell carcinomas. PGHS-2 protein could not be detected in 7 out of 9 samples of normal skin. The 2 remaining normal skin specimens, as well as 5 out of 6 actinic keratoses, the carcinoma in situ and 3 out of 6 squamous-cell carcinomas, showed a weak PGHS-2 signal. One actinic keratosis, the 3 other squamouscell carcinomas and the 2 keratoacanthomas contained moderate to very high levels of PGHS-2 protein. No PGHS-2 was found in the 4 malignant melanomas analyzed (Table III). In summary, PGHS-1 protein was found in all skin biopsies, whereas an expression of PGHS-2 could be demonstrated for actinic keratoses, the carcinoma in situ, squamous-cell carcinomas, keratoacanthomas and most basal-cell carcinomas, but not for malignant melanomas and the majority of normal skin samples.

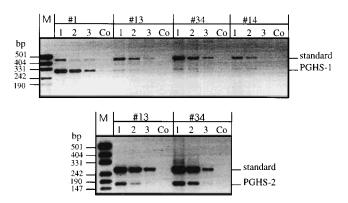


FIGURE 1 – RT-PCR analysis of PGHS-1 and PGHS-2 mRNA in biopsies from normal and neoplastic human skin. RNA samples from biopsies were mixed with PGHS-1 or PGHS-2 standard cRNAs and reverse-transcribed as described in Material and Methods. For the PCR reactions with PGHS-1- (top) and PGHS-2-specific primers (bottom), 40% (lane 1), 20% (lane 2) and 4% (lane 3) of the RT products or no template (Co) were used. The amplification products (top: PGHS-1, 304 bp; internal standard, 448 bp; bottom: PGHS-2, 184 bp; internal standard, 256 bp) were run in a 1.4% agarose gel, stained with ethidium bromide and photographed. M, DNA size marker; sample 1, normal skin; sample 13, actinic keratosis; sample 34, basal-cell carcinoma; sample 14, squamous-cell carcinoma. For identification of biopsies, see Table I.

TABLE II – ESTIMATION OF PGHS-1 AND PGHS-2 mRNA IN BIOPSIES FROM NORMAL AND NEOPLASTIC HUMAN SKIN $^{\rm I}$

Histology	Biopsy number	pg PGHS-1 mRNA/μg RNA	pg PGHS-2 mRNA/μg RNA
Normal skin	1	1800	nd
	2	108	4.6
	8	360	9.2
Actinic keratosis	12	18	92.0
	13	45	92.0
	14	18	4.6
	19	90	138.0
Squamous-cell carcinoma	25	45	6.9
•	26	90	46.0
Basal-cell carcinoma	34	18	9.2
	37	36	9.2
	38	45	9.2
	39	180	4.6

¹Amplification products of the internal standards and PGHS-1 and PGHS-2 targets as shown in Figure 1 were evaluated by densitometric analysis. The ratio of the PCR-amplified target amplicon to its simultaneously amplified standard amplicon was used to estimate the amount of target amplicon present in each sample RNA. mRNA levels were determined by multiplication with a correction factor for the different sizes of amplicon and authentic mRNA. nd, not determined.

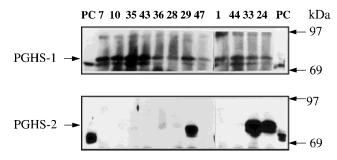


FIGURE 2 – Representative immunoblot analysis of PGHS-1 and PGHS-2 protein expression in normal human skin and in epidermal neoplasms. Homogenates from skin biopsies and the mouse keratinocyte cell line PDV (PC) were immunoprecipitated using rabbit anti-mouse PGHS-1-specific or rabbit anti-mouse PGHS-2-specific antibodies. The precipitates were electrophoresed and immunoblotted with the PGHS-1-specific (upper panel) or with the PGHS-2-specific anti-serum SC1745 (lower panel). The positions of the isozymes are indicated. Molecular weights of standard proteins are given in kilodaltons (kDa): normal skin, samples 1, 7 and 10; basal-cell carcinomas, samples 35, 36, 43 and 44; squamous-cell carcinomas, samples 24, 28 and 29; keratoacanthoma, sample 33; malignant melanoma, sample 47.

The intra-epithelial distribution of PGHS-1 and -2 in biopsies of normal human skin was investigated by means of immunohistochemistry. As shown in Figure 3, PGHS-1 protein was found throughout the epidermis, with increasing amounts in the suprabasal compartment of interfollicular epidermis as well as in the inner root sheath of the hair follicle (Fig. 3a,b). In addition, PGHS-1 was detected in endothelial cells of small vessels, in epithelial cells of the basal coil of an eccrine sweat gland and secretory ductal tubules (Fig. 3c,d). In contrast, staining for PGHS-2 protein was localized to few isolated keratinocytes in interfollicular skin, the hair follicle and sweat glands (Fig. 3g-i).

The result of the immunohistochemical analysis of neoplastic skin is shown in Figure 4 for actinic keratoses and the carcinoma *in situ*, and in Figure 5 for basal-cell and squamous-cell carcinomas. The 2 actinic keratoses displaying different degrees of dysplasia expressed PGHS-1 protein in the epidermis (Fig. 4a,e,f,i) and in areas of inflammatory cell invasion (Fig. 4b,f). Strong staining for PGHS-1 protein was also found for the carcinoma *in situ* (Fig. 4m-o). Intracellularly, the protein appears to be localized to the cytoplasm, to perinuclear membranes, and occasionally to the nucleus (Fig. 4a,e,o). PGHS-2 protein was also found in keratinocytes (Fig. 4d,g,h) with a cytoplasmic and perinuclear distribution (Fig. 4k) and in cells of the inflammatory infiltrate (Fig. 4c,h,j) as well as in dendritic cells (Fig. 4l). PGHS-2 specific signals were spread throughout the entire carcinoma *in situ*, with predominant localization in the stratum spinosum (Fig. 4p,q).

Figure 5 shows strong PGHS-1 expression throughout the squamous-cell and basal-cell carcinomas (Fig. 5a,g), as well as in regions of epidermis adjacent to basal-cell carcinomas (Fig. 5f). A strong signal is also seen in individual cells with dendritic morphology (Fig. 5h). PGHS-2 expression was spread over the whole section of the squamous-cell carcinoma (Fig. 5d). PGHS-2 immunosignals were observed in the basal-cell carcinoma (Fig. 5j,k) and in adjacent epidermis (Fig. 5i), as well as in cells of inflammatory infiltrates in the surrounding stroma (Fig. 5j,k).

DISCUSSION

Studies on rodent colon and mouse skin carcinogenesis imply that up-regulation of PGHS-2 expression is related to tumor development, and may indicate sensitivity to cancer prevention by NSAIDs (Müller-Decker *et al.*, 1995; Oshima *et al.*, 1996; Singh *et al.*, 1997). For humans, such a chemopreventive effect has already

TABLE III – PGHS-ISOZYME EXPRESSION IN NORMAL SKIN AND EPIDERMAL NEOPLASMS¹

Histology	Biopsy number	Microsomal protein (mg) ²	PGHS-1 protein	PGHS-2 protein
Normal skin	1	0.71	(+)	_
Normai skin	3	1.14	+	_
	4	1.08	+	(+)3
	5	1.46	+	$(+)^3$ $(+)$
	7	1.06	+	()
	8	0.80	(+)	_
	9	1.03	+	_
	10	1.22	+	_
	11	1.23	+	_
Actinic keratosis	15	0.18	+	(+)
retime keratosis	16	1.50	+	(+)
	17	0.21	+	(+)
	20	0.42	+	(+)
	21	1.50	+	(+)
	22	1.50	+	+
Carcinoma in situ	23	0.28	+	(+)
Squamous-cell carcinoma	24	1.59	+	++++
squamous con caromonia	27	0.76	+	(+)
	28	0.92	+	(+)
	29	1.49	+	+++
	30	0.21	+	+
	31	0.38	(+)	(+)
Keratoacanthoma	32	1.00	+	+
	33	2.20	+	++++
Basal-cell carcinoma	35	1.41	+	(+)
	36	1.00	+	(+)
	40	1.37	++	(+)
	41	1.09	+	(+)
	43	0.95	+	(+)
	44	2.40	+	
	45	1.01	++	(+)
	46	1.05	+	`
Malignant melanoma	47	0.30	+	_
2	48	1.30	+	_
	49	0.91	+	_
	50	0.30	(+)	_

¹Aliquots of homogenates from biopsies of 36 patients were first immunoprecipitated with an anti-PGHS-2 and subsequently with an anti-PGHS-1 anti-serum. Resolubilized precipitates were electrophoresed and immunoblotted with the corresponding anti-sera, with detection by enhanced chemiluminescence. The amounts of PGHS-1 and PGHS-2 proteins were graded as follows: ¬, no expression; (+), weak; +, moderate; ++, high; +++, ++++, very high.-²Amount of tissue homogenate used for immunoprecipitation.-³Positive after prolonged (3-fold) exposure of the X-ray film.

been firmly established for colorectal cancer (DuBois *et al.*, 1998), while PGHS-2 over-expression was also found in lung (Huang *et al.*, 1998), stomach (Ristimäki *et al.*, 1997) and breast-cancer tissue (Hwang *et al.*, 1998). The objective of our approach was to screen human skin cancer as another potential target for chemoprevention by NSAIDs, particularly PGHS-2-specific inhibitors. For this purpose, we investigated mRNA and protein expression and the cellular localization of PGHS-1 and PGHS-2 in biopsies from normal human skin and from pre-cancerous and cancerous lesions, using a collection of 50 skin biopsies.

Normal human skin was found to constitutively express PGHS-1 in all cell layers of the interfollicular epidermis and in the inner root sheath of the follicular epidermis. In addition, the epithelial lining of sweat glands and the endothelial cells of small blood vessels were found to be PGHS-1-positive, thus confirming the results of Leong *et al.* (1996). In contrast, PGHS-2-specific immunosignals were detected only in a few keratinocytes of the basal compartment of interfollicular epidermis and of more differentiated parts of the hair follicle. These data are consistent with the findings of Buckman *et al.* (1998), rather than with those of Leong *et al.* (1996), which showed relatively strong constitutive expression of both PGHS isozymes in human epidermis. The very low amounts of PGHS-2 mRNA in all biopsies of normal skin and of PGHS-2

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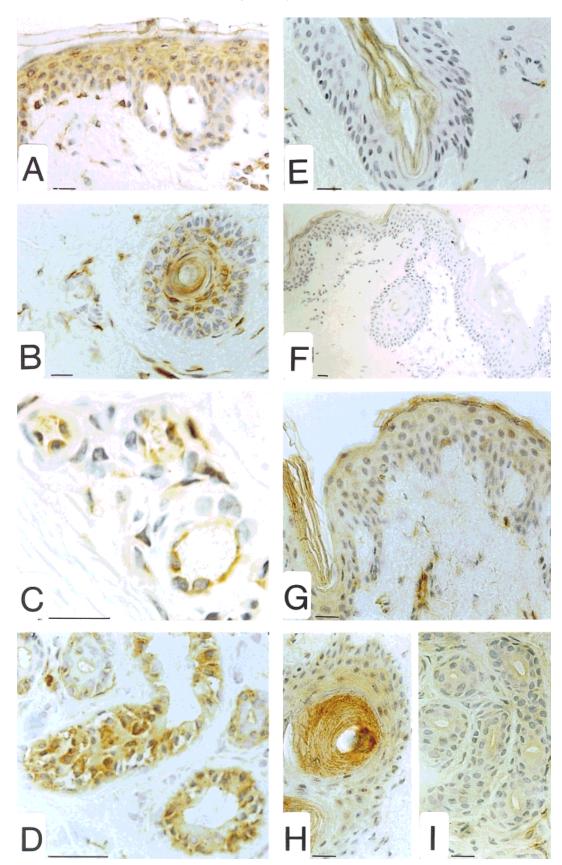


FIGURE 3 – Immunohistochemical localization of PGHS isozymes in sections of normal human skin. Cryostat sections (sample 6) were stained using the polyclonal goat anti-PGHS-1 anti-serum SC1754 (a-d) or the polyclonal goat anti-PGHS-2-peptide anti-serum SC1745 (g-i). (e) Anti-PGHS-1 anti-serum pre-adsorbed with PGHS-1 peptide; (f) primary anti-serum omitted. Scale bars = 25 μ m.

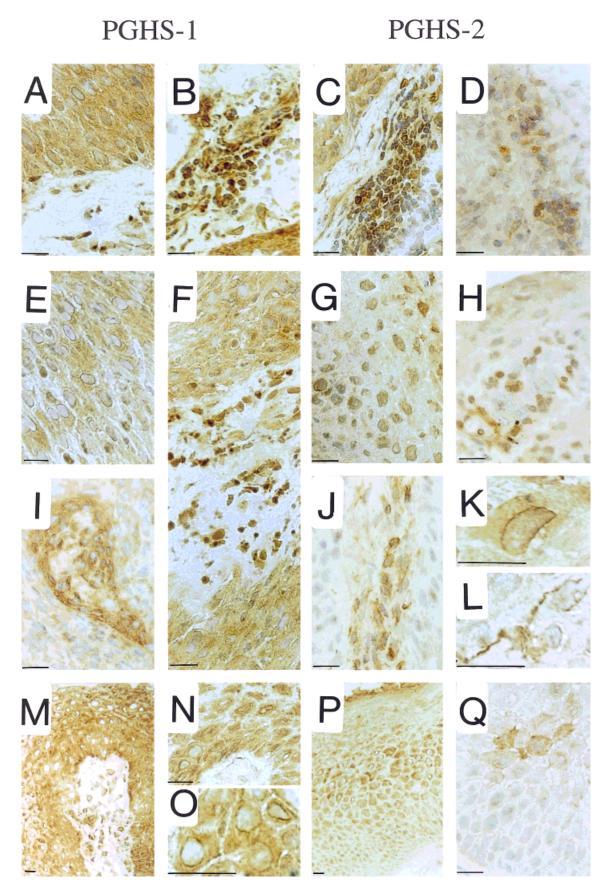


FIGURE 4 – Immunohistochemical localization of PGHS isozymes in pre-cancerous lesions of human skin. Cryostat sections were stained using the polyclonal goat anti-PGHS-1 anti-serum SC1754 (a,b,e,f,i,m-o) or the polyclonal goat anti-PGHS-2-peptide anti-serum SC1745 (c,d,g,h,j-l,p,q). (a-d) Actinic keratosis 20; (e-l) actinic keratosis 21; (m-q) carcinoma in situ 23. Scale bars = 25 μ m.

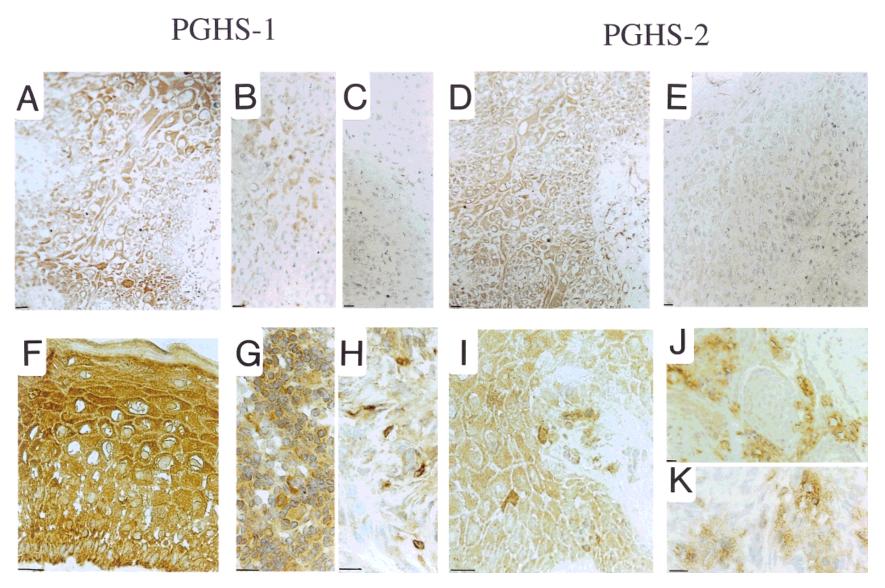


FIGURE 5 – Immunohistochemical localization of PGHS isozymes in malignant epidermal neoplasms. Cryostat sections were stained using the polyclonal goat anti-PGHS-1 anti-serum SC1754 (a,c,f-h) or the polyclonal goat anti-PGHS-2-peptide anti-serum SC1745 (d,e,i-k). (a-e) Squamous-cell carcinoma 29; (f-k) basal-cell carcinoma 42. (b) Anti-PGHS-1 anti-serum pre-adsorbed with PGHS-1 peptide; (c) primary anti-serum omitted; (e) anti-PGHS-2 anti-serum pre-adsorbed with PGHS-2 peptide. Scale bars = 25 μ m.

protein detected by immunoblotting in a few samples probably correspond to this scattered expression of PGHS-2 protein in isolated cells.

PGHS expression in neoplastic skin differed significantly from that in normal skin. Thus, the steady-state concentrations of mRNA for PGHS-1 were lower in actinic keratoses and squamous-cell carcinomas, whereas for PGHS-2 they were increased in 3 out of 4 actinic keratinoses and in 1 of the 2 squamous-cell carcinomas analyzed. In basal-cell carcinomas, PGHS-1 and -2 mRNA expression was similar to that in normal skin, while PGHS-2 protein was slightly but consistently increased. Large amounts of PGHS-2 protein were found in actinic keratoses, squamous-cell carcinomas and keratoacanthomas. For PGHS-1 protein no distinct differences were found between normal and neoplastic skin. This result appears to be in conflict with that of Leong et al. (1996), who detected no significant PGHS-1 expression in the single basal-cell carcinoma. High levels of PGHS-2 protein in squamous-cell carcinomas have been observed by others (Leong et al., 1996; Buckman et al., 1998). Malignant melanomas contained PGHS-1 protein but were PGHS-2-negative.

The immunohistochemical analysis of the tumor biopsies clearly demonstrated that, beside cells of the inflammatory infiltrate, dendritic cells and keratinocytes expressed both PGHS isozymes. PGHS-1 was found in more or less all tumor cells, being localized to the cytoplasm, the perinuclear membranes, and to the nucleus of keratinocytes. Extraordinarily strong staining for PGHS-1 was observed in the epidermis overlying an individual basal-cell carcinoma. PGHS-2 protein was minimal in basal-cell carcinomas, but was found to be spread throughout the actinic keratoses and squamous-cell carcinomas. Interestingly, the more dysplastic cells of actinic keratoses and of the carcinoma *in situ* were particularly stained by the PGHS-2 anti-serum.

Constitutive PGHS-2 expression in neoplastic skin was subject to large inter-individual variations. It nevertheless indicates that up-regulation of PGHS-2 is a consistent feature in the development of epidermal tumors, in particular of squamous-cell carcinomas. Since actinic keratoses are the precursor lesions to squamous-cell carcinomas (Brash, 1997), this up-regulation appears to be an early pre-malignant event in skin carcinogenesis. Actinic keratoses develop primarily on chronically sun-exposed areas of the body (Sober and Burstein, 1990). In fact, irradiation with UV-B, a constituent of sunlight, has been shown to induce transient expression of PGHS-2 in human skin (Buckman *et al.*, 1998).

A similar process is observed for chemical mouse-skin carcinogenesis according to the initiation-promotion protocol. Here, tumor-promoting stimuli, such as application of phorbol ester or skin wounding, transiently induce PGHS-2 (Scholz *et al.*, 1995), and the papillomas and carcinomas obtained by this approach constitutively over-express PGHS-2 (Müller-Decker *et al.*, 1995). Thus, in this animal model, the aberrant expression of PGHS-2 is clearly related to tumor promotion. This may be true also for UV carcinogenesis, since UV-B irradiation induces PGHS activity in mouse skin (Agarwal *et al.*, 1993) and has a strong tumor-promoting effect (Berg *et al.*, 1996). Taken together, our data and the results of other authors may justify a clinical trial aimed at the prevention of skin cancer in high-risk patients by the local or systemic administration of NSAIDs or even of specific PGHS-2 inhibitors.

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