Expression of Cyclooxygenase 2 in Human Malignant Melanoma

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ABSTRACT

Cyclooxygenase (COX)-2 is an inducible enzyme involved in production of prostaglandins in inflammatory processes. There is now increasing evidence that a constitutive expression of COX-2 plays a role in development and progression of malignant epithelial tumors. In the present study we investigated expression and function of COX-2 in malignant melanoma. Expression of COX-2 was determined by immunohistochemistry in 28 cases of primary skin melanoma and 4 benign nevi. We show that COX-2 was expressed in 26 cases (93%) of melanomas, with a moderate to strong expression in 19 cases (68%). Benign nevi as well as normal epithelium were negative in all cases. A constitutive expression of COX-2 mRNA and protein was found in five melanoma cell lines (A375, MeWo, SK-Mel-13, SK-Mel-28, and IGR-37) by using Northern blot as well as immunoblotting. All melanoma cell lines produced prostaglandin (PG) E₂ between 468 and 3500 pg/ml as determined by ELISA. Treatment with NS-398 (50 μM), a specific inhibitor of COX-2, suppressed PGE₂ production of all melanoma cell lines by 50–96%. The IC₅₀ for inhibition of PGE₂ production by NS-398 was determined as 4 μM, indicating that NS-398 acts via inhibition of the COX-2 isoenzyme. We could show that proliferation of melanoma cell lines was not influenced by treatment with NS-398 in concentrations up to 100 μM. However, NS-398 reduced Matrigel invasion of all five malignant melanoma cell lines by 50–68%. Our results indicate that COX-2 is expressed in malignant melanomas and may be involved in regulation of melanoma invasion. It remains to be investigated whether selective inhibitors of COX-2 might be useful for prevention or treatment of malignant melanoma.

INTRODUCTION

COXs are involved in control of inflammatory reactions and catalyze the conversion of arachidonic acid to PGH₂, which is the precursor of prostanooids. Two COX isoenzymes have been described: COX-1 is the constitutive form and is regarded as a housekeeping gene, whereas COX-2 is highly inducible by inflammatory stimuli (1). The role of COX-2 has been extensively studied in colorectal cancer, where this enzyme is expressed in adenomas and also is increased in carcinomas (2, 3). Epidemiological studies show that NSAIDs such as aspirin or sulindac reduce the incidence and mortality in colorectal carcinoma and several other types of cancer (4–7). Furthermore, in animal experiments inhibition of COX-2 reduced the incidence of colon carcinoma in rats treated with chemical carcinogens (8) as well as in APC knockout mice (9).

COX-2 is expressed in other carcinomas of the gastrointestinal tract as well, such as gastric or pancreatic adenocarcinomas (10). Additionally, an enhanced expression of COX-2 has been observed in well-differentiated hepatocellular carcinomas (11), well-differentiated adenocarcinomas of the lung (12), and in squamous carcinomas of the head and neck (13).

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The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; NSAID, nonsteroidal anti-inflammatory drug; TBS, Tris-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTZ, 3-(4,5-dimethylthiazole-2-yi)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; PPAR, peroxisome proliferator-activated receptor; APC, adenomatous polyposis coli.

Up to now, there is insufficient data supporting the function of COX-2 in tumors of nonepithelial origin (14). Thus, it is not clear whether COX-2-induced tumor progression is restricted to epithelial cancer or if it represents a more general mechanism of tumorigenesis. In the present study we investigated expression and function of COX-2 in malignant melanoma, a nonepithelial tumor characterized by a marked inflammatory stromal response.

MATERIALS AND METHODS

Materials. The human melanoma cell lines A375 (15), MeWo (16), SK-Mel-13 (17), SK-Mel-28 (18), and IGR-37 (19) were cultured in DMEM supplemented with 10% fetal bovine serum. NS-398 (Alexis, Grünberg, Germany) was dissolved in DMSO to a stock concentration of 100 mM. The cDNA fragment of human COX-2 as well as the mouse antihuman COX-2 monoclonal antibody used for immunoblotting was obtained from Cayman Biochemicals (Ann Arbor, MI). The monoclonal antibody against human COX-2 (clone33) used for immunohistochemistry was from Transduction Laboratories (Lexington, KY).

Immunohistochemistry. Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic purposes from 32 patients who underwent excision of skin tumors between 1992 and 1998 at the Department of Dermatology, Charité Hospital, Berlin. Twelve cases were melanomas within the horizontal growth phase (in situ and Clark level 1 or 2), whereas the other 16 cases were melanomas within the vertical growth phase (Clark level 3, 4, or 5). Four cases of benign nevus cell nevi were evaluated for comparison. Tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Routine H&E sections were performed for histopathological evaluation. Immunohistochemical staining was performed according to standard procedures. Briefly, slides were boiled in citrate buffer in a pressure cooker for 5 min and incubated with the monoclonal COX-2 antibody (1:2000; Transduction Laboratories) overnight at 4°C, followed by incubation with a biotinylated antimouse secondary antibody and the multilink biotin-streptavidin-amplified detection system (Biogenex, San Ramon, CA). Staining was visualized using a Fastred chromogen system (Immunotech, Hamburg, Germany). The intensity of the COX-2 immunostaining in tumor cells as well as surrounding inflammatory cells was evaluated independently by two pathologists and scored semiquantitatively as −, negative; +, weak; ++, moderate; and ++++, strong positive.

Immunoblotting. Cells grown to confluency in 50-mm Petri dishes were lysed in 100 μl of 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue. One hundred μg protein/sample were loaded on a 10% polyacrylamide gel. Proteins were separated by nondenaturing polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Immobilon-P, Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk for 2 h at room temperature and incubated overnight at 4°C with the monoclonal antibody (clone33) used for immunohistochemistry. Blots were probed with the polyclonal COX-2 antibody (1:5000; Transduction Laboratories) and then with a peroxidase-conjugated secondary antibody (Jackson Immunoresearch). Signals were visualized using an ECL detection system (Amersham). Membranes were exposed to X-ray films at −80°C. The expression of the housekeeping gene GAPDH was used as a control for equal protein loading.

Northern Hybridization. Total RNA was prepared with the RNeasy Kit (Qiagen, Hilden, Germany). RNA samples (5 μg) were electrophoresed in 1% agarose with 2.2 M formaldehyde and then blotted onto Hybond-N + membranes (Amersham, Braunschweig, Germany). After UV cross-linking (Hofer, San Francisco, CA), blots were hybridized in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) with [α-32P]cDNA probes using the megaprime DNA labeling system (Amersham). Blots were exposed to Kodak Biomax films at −70°C with intensifying screens. For standardization, hybridization with a cDNA probe for human GAPDH (Clontech) was performed.
**PGE₂ ELISA.** 1 × 10⁴ cells/well in 12-well plates were treated with or without 50 μM NS 398 (Alexis) in DMEM plus 10% FCS. After 24 h, the medium was replaced by 500 μl identical medium supplemented with 20 μM arachidonic acid (Sigma). The supernatants were harvested after 1 h and centrifuged at 5000 rpm for 10 min before blocking the COX by addition of 10 μg/ml indomethacin (Sigma). Samples were stored at −80°C. Concentration of PGE₂ in cell culture supernatants was determined using a specific ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The calculation of PGE₂ was estimated from the absorbance of the calculated standard curve. The lower limit of sensitivity for detection of PGE₂ was 36 pg/ml. The results are expressed as pg/ml per 10⁴ cells.

**Matrigel Invasion Assay.** Transwells with polycarbonate membranes (8-μm pores) in six-well tissue culture plates (Costar, Cambridge, MA) were coated with Matrigel (Becton Dickinson, Heidelberg, Germany) diluted in DMEM (1 mg/ml; 675 μl/4.7 cm²) and incubated for 60 min at 37°C. Afterward, membranes were washed once with DMEM. Cells were detached with trypsin/EDTA and seeded in the upper compartment of the transwell insert at a concentration of 2 × 10⁵ cells/ml serum-free culture medium. NS-398 (50 μM) or DMSO (0.05%) as control were added to the upper and the lower compartments. In some experiments, PGE₂ (0.05–100 μM) was added. After 72 h, cultures were incubated with 0.5 mg/ml MTT for 4 h, as described (20). Cells on the upper and lower surfaces of the transwell insert were removed separately, dissolved in DMSO, and measured using an ELISA reader. Metabolization of MTT by the noninvasive cells on the upper surface was found to be independent of treatment with NS-398 (data not shown) and was used to exclude an effect of the inhibitor treatment on cell proliferation and cell viability. Invasion was expressed as percentage of invasion compared with control (DMSO-treated) cells. To exclude an effect of NS-398 on MTT metabolization, NS-398 was added in some cases immediately before addition of MTT. This treatment did not change MTT metabolization (data not shown).

**Proliferation Assay.** Cell proliferation was measured using an XTT test (Boehringer-Mannheim, Mannheim, Germany). Cells were grown in 96-well plates for 3 days in medium containing 10% fetal bovine serum and NS-398 in concentrations between 0.1 and 100 μM. XTT metabolization was measured according to the manufacturer’s instructions. In additional experiments, cell proliferation was measured by direct cell counting using a CASY cell counter (Scha¨rfe Systems, Reutlingen, Germany). These experiments gave results similar to those of the XTT test (not shown).

**Statistics.** All data shown are from at least three independent experiments and are expressed as mean ± SE. The statistical significance was determined using Student’s t test or Fisher’s exact test. P < 0.05 was considered as significant. For statistical evaluation, the SPSS software Version 8.0 was used.

### RESULTS

**Expression of COX-2 Protein in Primary Tumors of Malignant Melanoma.** We have investigated 28 cases of primary malignant melanoma as well as 4 benign nevi for expression of COX-2 protein by immunohistochemistry (Table 1). Twelve cases of primary melanoma were within the radial growth phase (in situ; Clark level 1 or 2), whereas 16 cases were within the vertical growth phase (Clark levels 3–5). Expression of COX-2 in melanoma cells was found in 26 cases (93%), with moderate to strong intensity in 19 cases (68%). A granular cytoplasmic staining pattern was found in melanoma cells as well as in inflammatory cells within the stroma, such as macrophages or lymphocytes (Fig. 1). Adjacent normal epithelium including the melanocytes, the underlying connective tissue as well as the benign nevi were negative for COX-2 (Table 1, Fig. 1).

In nodular melanomas, there was a considerable heterogeneity of COX-2 expression, with an enhancement of COX-2 expression in the periphery of the tumor. The percentage of cases with a strong expression of COX-2 was higher in melanomas in the vertical growth (5 cases, 31%) than that in tumors in the radial growth phase (1 case, 8%). However, this relation was not statistically significant (Fisher’s exact test; P = 0.19). We did not observe any correlation between tumor thickness and COX-2 expression in tumor cells (Spearman’s rank correlation coefficient, 0.16) as well as stromal cells (correlation coefficient, −0.26). Thus, no significant differences in COX-2 expression in different stages of malignant melanoma were found.

**Expression of COX-2 mRNA and Protein in Malignant Melanoma Cell Lines.** For additional investigation of expression of COX-2 in malignant melanoma, we determined the expression of COX-2 mRNA and protein in five cell lines of malignant melanoma (MeWo, SK-Mel-13, SK-Mel-28, IGR 37, A375). As shown in Fig. 2, constitutive expression of COX-2 mRNA with a transcript of 4.5 kb was found in all cell lines. In Western blot analysis, all five melanoma cell lines expressed COX-2 protein with a size of Mr ~70,000 (Fig. 3). Expression levels of COX-2 mRNA and protein were comparable with the colon carcinoma cell line HT-29, which was used as a positive control (not shown).

**PGE₂ Production of Malignant Melanoma Cells.** As shown in Fig. 4, all cell lines produced PGE₂, with the highest production found in A375 cells (3500 ± 216 pg/ml) and the lowest in SK-Mel-13 cells (468 ± 18 pg/ml). In all cases, there was a strong reduction of PGE₂ production in cultures treated with NS-398 (Fig. 4). This reduction was maximal in A375 cells (96% inhibition by NS-398) and minimal in SK-Mel-28 cells (50% inhibition by NS-398). To estimate the IC₅₀ for inhibition of PGE₂ production by NS-398, we incubated MeWo cells with different concentrations of NS-398 (0.1–50 μM) and measured arachidonic acid-stimulated PGE₂ production (Fig. 5). We found a dose-dependent inhibition of PGE₂ production by NS-398, with an estimated IC₅₀ of 4 μM.

In additional experiments, we measured basal levels of PGE₂ in cells cultured for 24 h without further addition of arachidonic acid. In these unstimulated cultures, PGE₂ levels were above the detection limit of the ELISA were measured for all cell lines, ranging between
321 ± 28 pg/ml (IGR-37) and 138 ± 23 pg/ml (SK-Mel-13; data not shown). These basal levels of PGE2 were inhibited in all cell lines by NS-398.

Inhibition of Matrigel Invasion by NS-398. To evaluate the involvement of COX-2 in the invasion of malignant melanoma, a Matrigel assay with or without addition of NS-398 was performed. In all cell lines, invasion was reduced by NS-398 (50 μM; Fig. 6A). Inhibition of Matrigel invasion by NS-398 in different cell lines was between 50% (MeWo) and 66% (A375). To investigate the mechanism of regulation of melanoma invasion by COX-2, we measured Matrigel invasion after addition of exogenous PGE2. Exogenous PGE2 (0.05–100 nM) neither enhanced Matrigel invasion nor reduced invasion of MeWo, A375, IGR-37, SK-Mel-13, and SK-Mel-28 cell lines was between 50% (MeWo) and 66% (A375). To investigate the mechanism of regulation of melanoma invasion by COX-2, we measured Matrigel invasion after addition of exogenous PGE2. Exogenous PGE2 (0.05–100 nM) neither enhanced Matrigel invasion nor reduced
NS-398-induced inhibition of invasion of MeWo cells (Fig. 6B). Similar results were obtained for all other cell lines (data not shown).

**Proliferation of Malignant Melanoma Is Not Changed by NS-398.** We measured proliferation of the five malignant melanoma cell lines incubated with different concentrations of the specific COX-2 inhibitor NS-398. As shown in Fig. 7 for MeWo cells, no significant inhibition of cell proliferation was observed using concentrations of NS-398 between 0.1 and 100 μM. Similar results were obtained in three independent experiments for each of the five melanoma cell lines (data not shown).
For MeWo cells, we determined the IC\textsubscript{50} for PGE\textsubscript{2} inhibition by NS-398 as approximately 4 \(\mu\)M, which is within the range of the reported IC\textsubscript{50} of NS-398 for inhibition of COX-2 (1.77 \(\mu\)M; Ref. 31), whereas the IC\textsubscript{50} for inhibition of COX-1 by NS-398 is 75 \(\mu\)M (31). This suggests that the effect of NS-398 on melanoma PGE\textsubscript{2} production is mediated by inhibition of COX-2.

With regard to the function of COX-2 in melanoma biology, we did not find a regulation of melanoma growth by the COX-2 inhibitor NS-398. This is consistent with a recent publication, where it was shown that COX inhibitors did not reduce the size of mouse melanoma tumors (32).

We were able to demonstrate that NS-398 could inhibit invasion of malignant melanoma cells. The mechanisms involved in the inhibition of invasion by NS-398 are not completely clear thus far. A PGE\textsubscript{2}-mediated mechanism seems to be unlikely, because we have not been able to modulate melanoma invasion with exogenous PGE\textsubscript{2}. Furthermore, NS-398-induced suppression of invasion could not be overcome by addition of exogenous PGE\textsubscript{2}. This conclusion is additionally supported by the fact that the inhibitory effect on melanoma cell invasion was seen at relatively high concentrations of NS-398 (50 \(\mu\)M). We did not observe a significant inhibitory effect using lower concentrations of NS-398 (data not shown). This suggests that the inhibitory effect of NS-398 might be mediated by both COX-1 and COX-2, although we found only low level expression of COX-1 in inhibitory effect of NS-398 might be mediated by both COX-1 and COX-2. Thus, COX-2 expression is upregulated in human pancreatic cancer. Cancer Res., 58: 409–412, 1998.


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