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Inhibitory effect of acetylsalicylic acid on metalloproteinase activity in human lung adenocarcinoma at different stages of differentiation

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Abstract

The mechanism underlying the anticancer effect of nonsteroidal anti-inflammatory drugs (NSAIDs) is not clear. We addressed the question whether the alterations in collagen content in lung adenocarcinomas reported in previous studies result from dysregulation of gelatinolytic activity and whether the activity is altered by acetylsalicylic acid in vitro. Human lung adenocarcinomas were divided into three groups: well-differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) tumors. Each group was compared with normal lung tissue with respect to tissue collagen and collagen degradation product content (hydroxyproline assay), gelatinolytic activity (zymography) and the expression of matrix metalloproteinases, MMP-2 and MMP-9 (Western immunoblot). Moreover, in the studied tissues, the effect of acetylsalicylic acid on gelatinolytic activity was measured. The lung adenocarcinoma G1 had a similar collagen content as normal lung tissue but increased amounts of collagen degradation products and free hydroxyproline. These phenomena were accompanied by a marked increase in gelatinolytic activity (MMP-2 and MMP-9) in the G1 tumor. In adenocarcinoma G2, the free hydroxyproline content and gelatinolytic activity were increased, while the collagen and collagen degradation product contents were not markedly altered, compared to control. In contrast, adenocarcinoma G3 had an increased tissue collagen content (by about 60%), decreased percentage of collagen degradation products and similar gelatinolytic activity, compared to normal lung. Acetylsalicylic acid was found to inhibit gelatinolytic activity both in control and adenocarcinoma tissues, preferentially the active forms of gelatinases MMP-2 and MMP-9. The results suggest that human lung adenocarcinoma G1, through an elevated expression of the activated forms of both MMP-2 and MMP-9, may represent a more invasive phenotype than less differentiated tumors G2 or G3. It indicates that lung adenocarcinoma G1 should be considered as a possible target for metalloproteinase inhibitory therapy. Acetylsalicylic acid may be such a therapeutical agent in cancer prevention or early stages of tumor growth.

Keywords: Acetylsalicylic acid; Metalloproteinase; Adenocarcinoma

1. Introduction

Acetylsalicylic acid and other nonsteroidal anti-inflammatory drugs (NSAIDs) appear to have antineoplastic properties that are most prominent in the digestive tract, especially the large bowel (Baron, 1995). The drug prevents colorectal polyps from forming and becoming colon cancer. Epidemiological data also suggest possible protective effects in the stomach and esophagus (Baron and Sandler, 2000). Acetylsalicylic acid, the most common of the NSAIDs, may also inhibit the growth and multiplication of animal and human lung adenomas. The drug effect was examined at the level of prostaglandins in lung tumors in mice and in cultured human non-small-cell lung tumor cells (Nelson, 1995). However, the mechanism of the anticancer action of NSAIDs is not understood.

One of the consequences of neoplastic transformation is an aberration of the biosynthesis of some proteins of the extracellular matrix, mainly fibronectin and type I collagen (Hynes, 1976; Slack et al., 1992). Type I collagen is the most abundant extracellular protein of vertebrates, predominantly produced by fibroblasts, but also synthesized and secreted by a variety of differentiated cells (Bornstein and Sage, 1989). The interaction between cells and extracellular matrix proteins, e.g. collagen, can regulate cellular gene expression, and differentiation, cell growth (Bissel, 1981; Carey, 1991) and can play an important role in tumorigenicity and invasiveness (Ruoslahti, 1992). It has been shown that most normal and neoplastic cells recognize many extracellular proteins by means of specific cell surface receptors, called

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integrins. They are thought to be important for tumor cell attachment, migration, proliferation, progression and survival (Albelda and Buck, 1990).

Inavasion by neoplastic cells has been considered as a three-step process (Liotta et al., 1991): attachment of the cells to the extracellular matrix, pericellular degradation of the extracellular matrix, and tumor cell movement into the proteolytically modified matrix. Invasive cells secrete a variety of hydrolases which may change the extracellular matrix composition (Chen, 1992) and may also modulate the extracellular matrix by a variety of nondegradative mechanisms (Dedhar and Saulnier, 1990; Zetter, 1993). Destruction of the basement membrane is an important step in the invasion and metastasis of cancer (Nicolson and Poste, 1982). Therefore, tumor progression might critically depend on the breakdown of collagen and other extracellular matrix proteins. Matrix metalloproteinases, in particular the MMP-9 (92-kDa) and MMP-2 (72-kDa) gelatinases, have been implicated in the progression of breast, colorectal and gastric carcinomas, and non-small-cell lung carcinoma (Brown et al., 1993a,b). These enzymes are believed to be responsible for the degradation of tissue basement membrane, the breakdown of which is an early feature of malignant disease.

Lung adenocarcinoma contains subpopulations of cancer cells with widely differing metastatic potential. It is believed that the poorly differentiated adenocarcinoma has a higher metastatic potential. However, there is no convincing data in the literature to support such a hypothesis.

The current study was therefore undertaken to characterize gelatinolytic activity in lung adenocarcinomas at various stages of differentiation and to evaluate the effect of acetylsalicylic acid on gelatinolytic activity in these tissues.

2. Materials and methods

2.1. Materials

Acetylsalicylic acid, gelatin, dimethyl sulfoxide (DMSO), L-hydroxy-proline, Triton X-100, anti-mouse immunoglobulin G (whole molecule) alkaline phosphatase conjugate antibody, Sigma-Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) reagent and most other chemicals and buffers used were provided by Sigma, USA. Monoclonal anti-human matrix metalloproteinases MMP-2 and MMP-9 antibodies were purchased from R&D Systems, Germany. Nitrocellulose membrane (0.2 μm), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards and Coomassie brilliant blue R-250 were received from Bio-Rad Laboratories.

2.2. Histology

Thirty-six human lung carcinomas and twelve representative samples of normal lung parenchyma (matched uninvolved tissue) obtained from the margins of surgically resected tumors were selected from the multi-files of the Department of Pathology Medical Academy in Bialystok, Poland. The histologic type of the tumors was determined according to the classification of lung tumors (Sobin and Yesner, 1981). The adenocarcinomas were divided into three groups, according to the degree of differentiation: well-differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) tumors.

2.3. Preparation of tissue extracts

Lung tissues were placed in cold-ice 0.05 mol/l Tris–HCl, pH 7.6 buffer and extensively perfused with the same buffer. The tissues were stored at −70 °C, until assay. The tissue homogenates (20% w/v) were prepared in 0.05 mol/l Tris–HCl, pH 7.6 with the use of a homogenizer (Polytron) and subsequently were sonicated at 0 °C. Homogenates were centrifuged at 16,000 × *g* for 30 min at 4 °C. Supernatant (tissue extract) was used for zymography assay and Western immunoblot analysis.

2.4. Separation of intact tissue collagen from collagen degradation products

The method of Kang et al. (1967) was used to separate collagen degradation products from intact tissue collagen. Tissue homogenate (20% w/v) was incubated with an equal volume of 1 mol/l acetic acid for 2 h at room temperature. Intact collagen was precipitated by the addition of sodium chloride at a final concentration of 2.5 mol/l and centrifuged at 16,000 × *g* for 15 min at room temperature. The supernatant containing collagen degradation products was hydrolyzed in 6 mol/l hydrochloric acid for 16 h at 124 °C and hydroxyproline was determined.

2.5. SDS-PAGE

Slab SDS/PAGE was used according to the method of Laemmli (1970). Samples of cell supernatants (20 μg of protein) were electrophoresed. The following Bio-Rad unstained high-molecular-weight standards were used: galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa).

2.6. Zymography

Gelatinolytic activity was determined according to the method of Unemori and Werb (1986). Tissue extract was mixed with Laemmli (1970) sample buffer containing 2.5% SDS (without reducing agent). Equal amounts (about 20 μg) of protein were electrophoresed under nonreducing conditions on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin. After electrophoresis, the gels were incubated in 2% Triton X-100 for 30 min at 37 °C to remove SDS and incubated for 18 h at 37 °C in substrate buffer (50 mM Tris–HCl buffer, pH 8, containing 5 mM $CaCl_2$). After

staining with Coomassie brilliant blue R-250, gelatin-degrading enzymes present in tissue extract were identified as clear zones against a blue background.

2.7. Western blot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2-μm-pore size nitrocellulose at 100 mA for 1 h by using an LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with monoclonal anti-human MMP-2 or MMP-9 antibodies at a concentration of 1 μg/ml in 5% dried milk in Tris-buffered saline/Tween-20 (TBS–T, 20 mmol/l Tris–HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween-20) for 1 h. In order to analyze MMP-2 and MMP-9, alkaline phosphatase-conjugated antibody against mouse IgG (whole molecule) was added at a concentration of 1:7500 in TBS–T. Incubation was continued for 30 min with slow shaking. Then in both cases, nitrocellulose was washed with TBS–T (five times for 5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

2.8. Determination of protein and hydroxyproline

Protein was determined according to the method of Lowry et al. (1951). Hydroxyproline was determined by the method of Prockop and Udenfriend (1960).

2.9. Statistical analysis

In all experiments, the mean values for 12 assays ± stan-standard deviations (S.D.) were calculated. The results were analyzed by two-way analysis of variance between groups (ANOVA) test, taking $P < 0.05$ as significant.

3. Results

We measured the content of collagen in the studied tissues by means of the hydroxyproline assay. The tissue proteins were hydrolyzed with 6 mol/l hydrochloric acid and released hydroxyproline was determined. The lung adenocarcinomas G1 and G2 had a similar hydroxyproline content as normal lung. Adenocarcinomas G3, however, had a significantly ($P < 0.05$) increased total hydroxyproline content (Table 1). Because some of the hydroxyproline may have been derived from collagen degradation products, these products were separated as described in Materials and methods, hydrolyzed and released hydroxyproline was determined. The free hydroxyproline content in the tissues was also measured. As can be seen from Table 1, the free hydroxyproline content in lung adenocarcinomas was significantly ($P < 0.05$) elevated compared to that in normal lung; however, the amount of hydroxyproline derived from collagen degradation products was not significantly different. Comparison of the amount of total hydroxyproline in tissues and the amount of collagen degradation product-derived hydroxyproline made it possible to calculate the percentage of collagen degradation products in tissues. The results presented in Table 1 show that about one third of normal lung hydroxyproline was derived from collagen degradation products, whereas in lung adenocarcinoma G1, collagen degradation product-derived hydroxyproline accounted for a much higher proportion. In the case of adenocarcinomas G2 and G3, collagen degradation product-derived hydroxyproline represented a much lower percentage of the total hydroxyproline content compared to that in control tissue. The lower the differentiation of lung adenocarcinomas, the lower the percentage of degraded collagen.

Since disturbances in collagen degradation are accompanied by dysregulation of tissue gelatinase activity, we determined the tissue gelatinolytic activity by zymography. As can be seen from Fig. 1A (lane 1), normal lung parenchyma contained three main gelatinases: "a" represented by a protein of 106 kDa; "b" represented by a protein of 92 kDa (MMP-9); "d" represented by a protein of 72 kDa (MMP-2). Gelatinases "b" and "d" are well-defined tissue metalloproteinases (Brown et al., 1993a,b). In adenocarcinomas G1 (lane 2), there was an increase in the activity of most gelatinases, compared to that in normal lung parenchyma. Furthermore, adenocarcinoma G1 contained a 82-kDa gelatinase "c"—presumably an active form of the latent 92-kDa gelatinase and 62-kDa gelatinase "e"—presumably an active form of latent 72-kDa gelatinase.

In adenocarcinoma G2 (Fig. 1A, lane 3), the activity of gelatinases "b", "c", "d" and "e" was elevated, similarly as in adenocarcinoma G1. However, only trace activity of

Table 1
Total, free and collagen degradation products (CDP)-derived hydroxyproline in normal lung parenchyma and lung adenocarcinomas of various degrees of differentiation

Groups	Degree of differentiation	*n*	Hydroxyproline μg/mg protein			Percentage of CDP-derived hydroxyproline
			Total	Free	CDP derived	
Normal lung		12	16.9 ± 2.1	0.45 ± 0.06	5.5 ± 1.5	32.5
Adenocarcinoma	G1	12	16.5 ± 1.8	0.80 ± 0.10[a]	7.2 ± 1.2	43.6
	G2	12	19.8 ± 2.6	2.6 ± 0.25[a]	4.6 ± 1.2	23.2
	G3	12	27.3 ± 4.7[a]	1.65 ± 0.25[a]	4.4 ± 1.1	16.3

n = number of samples.
[a] $P < 0.05$.

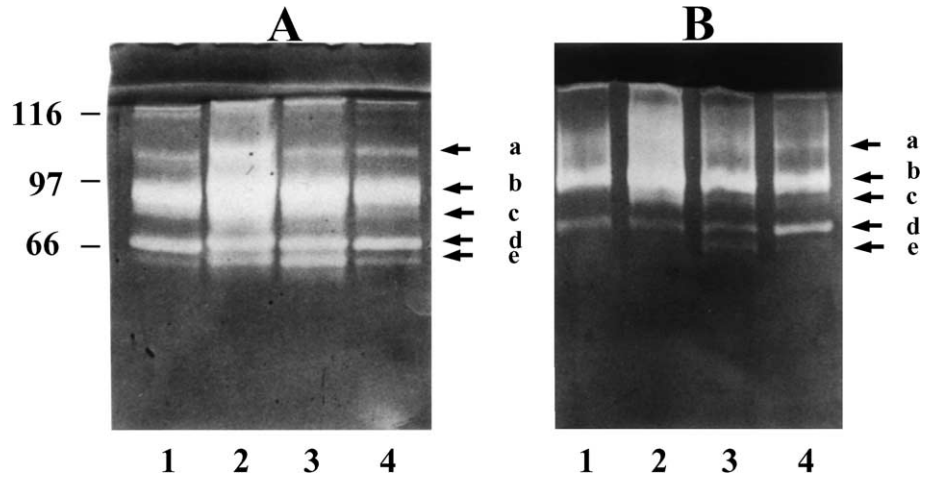

Fig. 1. Gelatinolytic activity in intact (A) and acetylsalicylic acid (5 mM)-treated (B) extracts of normal lung parenchyma (lane 1) and lung adenocarcinomas: G1 (lane 2), G2 (lane 3), G3 (lane 4); (a) 106-kDa gelatinase, (b) 92-kDa gelatinase, (c) 82-kDa gelatinase, (d) 72-kDa gelatinase, (e) 62-kDa gelatinase. The same amount of supernatant protein (20 μg) of pooled homogenate extracts from 12 control and 12 carcinoma tissues was run in each lane.

gelatinase "a" was detected, as in control tissue. In adenocarcinoma G3 (Fig. 1A, lane 4), a similar activity of all designated gelatinases was seen as in control lung tissue.

Previously, it has been found that acetylsalicylic acid suppresses the invasion and metastasis of some tumors, by inhibiting MMP-9 (Murono et al., 2000). Therefore, we determined the effect of acetylsalicylic acid on gelatinolytic activity in control lung and lung adenocarcinomas. The acetylsalicylic acid was neutralized with sodium hydroxide, buffered in 0.05 mol/l Tris–HCl, pH 7.6, and added to tissue extract. Gelatinolytic activity was determined in tissue extracts treated with 5 mmol/l acetylsalicylic acid, for 15 min at room temperature. As can be seen from Fig. 1B, the investigated drug induced a decrease in the activity of all designated gelatinases both in control and adenocarcinoma extracts. More importantly, the active forms of gelatinases "c" and "e" were almost completely inhibited. This suggests that acetylsalicylic acid inhibits gelatinolytic activity at different stages of human lung adenocarcinoma differentiation. However, the most pronounced effect was observed in G1 and G2 tumors. The gelatinases that are targets for acetylsalicylic acid inhibitory activity are represented by MMP-2 and MMP-9. As can be seen from Fig. 2, Western immunoblot analysis (with specific antibodies against metalloproteinases MMP-2 and MMP-9) revealed the presence of both MMP-2 and MMP-9 in control lung tissue and in the adenocarcinomas.

No distinct differences in their amount in control and adenocarcinoma tissues were found after treatment with acetylsalicylic acid (Fig. 2).

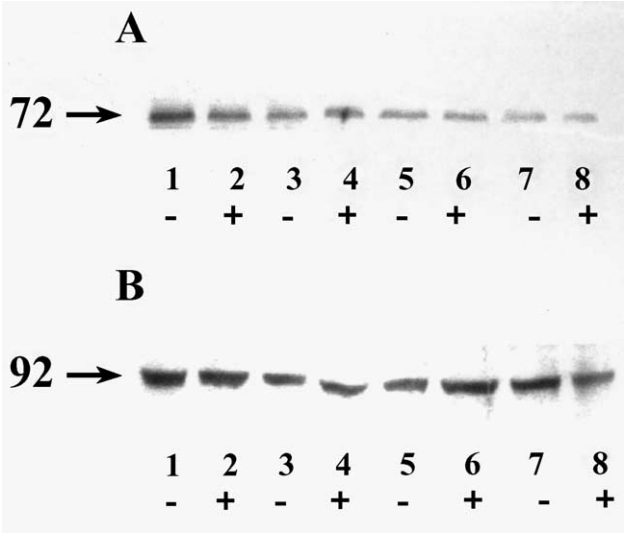

Fig. 2. Western immunoblot analysis for MMP-2, 72-kDa gelatinase (A) and MMP-9, 92-kDa gelatinase (B) from normal lung parenchyma extract (lanes 1 and 2) and lung adenocarcinomas: G1 (lanes 3 and 4), G2 (lanes 5 and 6), G3 (lanes 7 and 8). The symbols "+" and "–" indicate the presence or absence of 5 mmol/l acetylsalicylic acid in the tissue homogenates, respectively. The same amount of supernatant protein (20 μg) of pooled homogenate extracts from 12 control and 12 carcinoma tissues was run in each lane.

4. Discussion

The best-studied consequence of metastatic invasion by malignant cells is an increased expression of extracellular matrix-degrading enzymes (Mccawley and Matrisian, 2000). In respect to collagen, the proteinases that can initiate its breakdown are tissue metalloproteinases (MMPs). Increased expression of gelatinases, including MMP-2 and MMP-9, during neoplastic transformation has been shown by a number of workers (Nakajima and Chop, 1991). However, the expression does not correlate with gelatinolytic activity. Activation of gelatinases from zymogen precursors occurs via limited cleavage by other proteases, while suppression of their activity is accomplished by a variety of plasma and tissues inhibitors (Nakajima and Chop, 1991). The technique of zymography offers a simple, yet sensitive, means of

resolving the activated species from latent proenzyme and thereby provides additional data on the relationship between metalloproteinase activity and possible tumor progression. Since the conversion of the latent matrix metalloproteinases to activated species involves the proteolytic removal of a 10-kDa amino-terminal domain, the latent and activated forms can be resolved on the basis of size. Following separation, both latent and active species are restored to conformations that display gelatinolytic activity (Birkedal-Hansen and Taylor, 1982). The latent species displays gelatinolytic activity because of the artificial conditions of the zymographic process. For instance, the 72-kDa species would be inactive under physiological conditions.

Using this technique, we found an increased gelatinolytic activity in adenocarcinomas G1 and G2 (but not in G3) compared to control parenchyma. While in control tissue we detected mostly the latent gelatinases, in adenocarcinoma G1 and G2, also active forms of the metalloproteinases were found. The activity is probably due to MMP-9 and MMP-2, as shown by Western immunoblot analysis. The increase in the MMP activity was however not accompanied by an increase in the amount of specific enzyme protein. This phenomenon is probably due to the fact that monoclonal antibodies against MMP-9 and MMP-2 (used in this study) recognize only latent forms of the enzymes. It suggests that post-translational mechanisms may be responsible for increase in the MMP activity.

The result was corroborated by data showing an increase in free hydroxyproline and collagen degradation product contents in adenocarcinoma G1, compared to normal lung parenchyma. Interestingly, with respect to gelatinolytic activity, adenocarcinoma G3 showed similar patterns as control tissue. This may suggest that adenocarcinoma G1 may represent a phenotype with a higher metastatic potential than adenocarcinoma G3.

The data presented here show that a commonly used anti-inflammatory drug, acetylsalicylic acid, induces in vitro a decrease in gelatinolytic activity in both control and lung adenocarcinoma tissues. The inhibition was directed mostly against the active forms of MMP-9 and MMP-2. No distinct differences in the expression of their latent forms were found in tissues treated with acetylsalicylic acid. The possible mechanism may involve direct inhibition by acetylsalicylic acid of active forms of MMPs.

The concentration of acetylsalicylic acid used in these studies was rather high compared with the pharmacological doses of the drug recommended for the therapy of inflammation. Lower doses of the drug (2 mM, which is within the therapeutic plasma level for anti-inflammatory action) resulted in less inhibition of gelatinase activity under our experimental conditions (data not shown). Our data are consistent with some other studies showing that higher than pharmacological doses of acetylsalicylic acid may prevent tumor growth (Nelson, 1995; Rotem et al., 2000). Our previous studies on prolidase activity in adenocarcinoma tissues suggested that the enzyme elevation reflects the degree of differentiation of the tissue (Karna et al., 1997). In other studies, we have shown that prolidase activity can be inhibited by acetylsalicylic acid (Miltyk et al., 1996). At 5 mM concentration, the drug induced 50% inhibition of the enzyme in human skin fibroblasts. Whether prolidase contribute to neoplastic phenotype of adenocarcinoma tissues is being presently studied in our laboratory. Nevertheless, the above data explain the reason for study this drug at 5 mM concentration. We studied also adenocarcinoma tissues that had been incubated with lower concentrations of acetylsalicylic acid for more than 15 min. Lower concentrations of the drug produced much less inhibition of gelatinolytic activity. Prolonged incubation of tissue homogenate (without the drug) resulted in the disappearance of bands corresponding to the active forms of gelatinases and a decrease in bands corresponding to their latent forms, presumably due to an increase in their degradation by other proteases present in the tissue homogenate. Therefore, the described conditions were found optimal for the assay.

It is known that acetylsalicylic acid inhibits cell invasiveness induced by Epstein–Barr virus latent membrane protein 1 through suppression of MMP-9 expression (Murono et al., 2000). The mechanism underlying the chemopreventive effect of NSAIDs on tumor growth (Nelson, 1995; Rotem et al., 2000) may therefore involve the inhibition of both metalloproteinase expression and activity.

These results indicate that lung adenocarcinoma may be considered a possible target for metalloproteinase inhibitory therapy, and acetylsalicylic acid or perhaps other NSAIDs may represent therapeutic agents for potential application in cancer prevention or early stages of tumor growth.

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