Homocysteine is a potent inhibitor of human tumor cell gelatinases

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Abstract

Extracellular matrix-degrading gelatinases are mainly involved in tumor invasion and metastasis. Previous experimental data from our group and others suggested that homocysteine could have a potential modulatory role on the proteolytic balance at the extracellular matrix. Therefore, we studied the effects of homocysteine on extracellular matrix-degrading proteases using model human tumor cell lines and a combination of in vitro fluorogenic assay and zymographic techniques. Homocysteine is shown to be the thiol compound with the most potent inhibitory activity on matrix metalloproteinase 9. Zymographies reveal that matrix metalloproteinase 2 is, at least, as sensitive to inhibition by homocysteine as matrix metalloproteinase 9 is. This study opens new ways to the potential pharmacological use of thiol compounds.

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Homocysteine is a sulfur-containing, non-proteinogenic amino acid biosynthesized from methionine which takes a key place in common in between folate cycle and activated methyl cycle [6]. An imbalance of homocysteine metabolism has been associated with several disorders and diseases [6–9]. Recently, homocysteine has been shown to induce the expression and synthesis of the tissue inhibitor of metalloproteinases-1 (TIMP-1) in a variety of cells [10]. In addition, exposure of cultured endothelial cells to homocysteine reduces cell proliferation, increases the amount of telomere length lost per population doubling, and increases the expression of cell surface molecules linked to vascular diseases such as intracellular adhesion molecule 1 and plasminogen activator inhibitor 1 [11–14]. Very recently, homocysteine has been shown to inhibit angiogenesis in vitro and in vivo [15,16]. These findings suggest that homocysteine may alter extracellular matrix homeostasis on diverse tissue backgrounds.

The purpose of the present study was to examine the effects of homocysteine on extracellular matrix-degrading proteases using human tumor cell lines. Herein, we demonstrate that, in fact, homocysteine strongly inhibits human tumor cell gelatinases (matrix metalloproteinases 2 and 9).
Materials and methods

Cell culture. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (4.5 g/L) and glutamine (2 mM), supplemented with the antibiotics penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphotericin (1.25 μg/mL) and with 10% fetal bovine serum.

Fluorogenic assay of MMP-9 activity. Purification of HT-1080 tumor cell MMP-9 protein was carried out as described elsewhere [17]. Prior to their use for the in vitro assay of activity, samples of purified MMP-9 were completely preactivated by treatment with 1 mM 4-amino-phenylmercuric acetate (APMA) for 12 h at 37°C. We used a fluorogenic assay by using a peptide substrate (supplied by Bachem) highly specific for this gelatinase, namely, 2,4-dinitrophenyl-Pro-[β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-(N-Me-Abz)]-NH2. This substrate is cleaved by MMP-9, releasing 2,4-dinitrophenol, the quenching group of the molecule, and N-methyl-anthranylic acid (N-Me-Abz), the fluorogenic group. Previous titration of the activity of our purified samples of MMP-9 made us to choose 5% (v/v) MMP-9 solution, 5 μM peptide substrate, and different concentrations of thiols in a final volume of 100 μL assay buffer. Activity was followed for 30 min at 37°C in a fluorescence plate reader FL600FA (Bio-Tek, Winooski, VT) at 360/460 nm excitation/emission set, a gain of 100 and bottom lecture mode. Samples with 20 mM EDTA added were used as positive controls of inhibition.

Conditioned media and zymography for the gelatinolytic activity of MMP-2 and MMP-9. Conditioned media were prepared as previously described [18,19]. The gelatinolytic activity of MMP-2 and MMP-9 delivered to the conditioned media by the different tumor cells was detected by using gelatin zymography, as follows. Samples were subjected to non-reducing SDS/PAGE with gelatin (1 mg/mL) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mM Tris–HCl, pH 7.4, supplemented with 2% Triton X-100, and twice with 50 mM Tris–HCl, pH 7.4. Each wash was for 10 min and with continuous shaking. After the washes, the gels were incubated at 37°C for 12–16 h immersed in a substrate buffer (50 mM Tris–HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mM CaCl2, and 0.02% Na3N). Afterwards, the gels were stained with Coomassie blue R-250 and the bands of gelatinase activity could be detected as non-stained bands in a dark, stained background. Alternatively, to detect direct effect of homocysteine and other thiols on the in situ gelatinolytic activity of samples, different concentrations of homocysteine and other thiols were added to the substrate buffer during the incubation of gels containing control conditioned media. Quantitative analysis was carried out by using the NIH Image 1.6 program.

Results and discussion

An essential step in tumor invasion and metastasis (but also in angiogenesis) is a proteolytic degradation of extracellular matrix [2,3,20–22]. In fact, a positive correlation among tumor invasiveness and extracellular matrix protease levels has been shown [4,23].

Homocysteine is one of the main circulating thiols in humans, with plasma levels higher than those of glutathione (4 μM) in healthy individuals and even higher than those of cysteine (200 μM) in cases of severe hyperhomocysteinemia [7,24]. Based on recent findings by other groups, we recently postulated that homocysteine could be involved in the modulation of angiogenesis and tumor invasion [6]. We have tested such a hypothesis and, in fact, we have previously shown that homocysteine exerts anti-angiogenic effects on cultured endothelial cells [16] and anti-invasive effects on tumor cells [19]. Since tumor invasion and metastasis require remodeling of extracellular matrix, and gelatinases (MMP-2 and 9) are mainly involved in this remodeling [2,3], we investigated whether homocysteine can inhibit both activities. We also compared the effects of homocysteine with those of other thiols, since the pharmacological potential of thiols as inhibitors of gelatinases has been previously shown [25,26].

Fig. 1 shows that while cysteine, glutathione, N-acetyl-cysteine and 2-mercaptoethanol completely inhibited MMP-9 activity only at concentrations in the mM range, homocysteine behaved as a much more potent inhibitor, abolishing MMP-9 activity at a concentration...
as low as 75 μM. At 0.1 mM, the potent non-physiological reductant agent 2-mercaptoethanol only inhibited MMP-9 activity by 60 ± 5%, whereas the physiologically relevant thiols, cysteine and glutathione produced no significant inhibition of MMP-9 activity (12 ± 13% and 22 ± 12%, respectively). These data show that homocysteine seems to be the most potent thiol inhibitor of tumor gelatinases, probably due to its higher chemical reactivity as compared with other naturally occurring thiols [27]. It must be stressed that complete inhibition of MMP-9 activity is achieved at homocysteine concentrations almost 300-fold lower than those required to achieve this effect by using other thiols.

Homocysteine indeed inhibits gelatinases by directly blocking enzymatic activity. This block could be caused by a competitive affinity of homocysteine for the zinc ion necessary for the enzymatic activity of gelatinases [28]. An activation of pro-MMP-2 by low concentrations of homocysteine has been demonstrated previously [29]. This activation is claimed to be in accordance with the “cysteine-switch” mechanism [30], occurring without further autoproteolysis of the enzyme. These previous data are not in contradiction with our own data, since our in vitro assays were done on APMA-preactivated MMP-9 purified enzyme. In fact, the same previous study shows that APMA-activated pro-MMP-2 can be inhibited by homocysteine [29].

Both HT-1080 and human osteosarcoma U2-OS cells produce and release both gelatinases, but HT-1080 releases more MMP-9 than MMP-2 and U2-OS releases more MMP-2 than MMP-9 [18]. To show whether this inhibitory effect of homocysteine is also exerted on MMP-2, conditioned media from U2-OS cells were used for gelatinolytic assays where the thiol was added to substrate buffer. In this assay, 0.1 mM homocysteine did not produce any significant effect either on the MMP-2 and the MMP-9 proteolytic bands (results not shown). Fig. 2 shows that 5 mM homocysteine, glutathione or cysteine added to the substrate buffer completely abolished the in situ reconstitution of gelatinase activity in zymographic assays. Another thiol compound, namely, 2-mercaptoethanol only partially inhibited the gelatinases and the reduced compound NADH had no effect.

Conditioned media from both tumor cell lines exposed for 24h to 5 mM homocysteine contained much lower MMP-2 and MMP-9 levels than control, untreated cells, as determined by gelatin zymography (Fig. 3 and Table 1). Although the homocysteine concentration here used in cell culture experiments is rather high as compared with the concentrations in plasma under physiological and pathological conditions, this is unavoidable because cells in culture upregulate the metabolism of homocysteine considerably. It has been recently shown that such high extracellular concentrations of homocysteine are required to observe cellular responses to homocysteine treatment [12]. In fact, similar high concentrations of homocysteine have been used in recent relevant studies involving cultured endothelial cells [11–16]. However, the known limitations of cell cultures make us to be cautious regarding the actual physiological relevance of our data. Further studies on in vivo models would be necessary to elucidate this issue.

Modulation of extracellular and intracellular thiols is being investigated as a promising strategy in cancer prevention [31]. Other mechanisms related to its redox status are likely to contribute to these effects. Further studies in this direction are warranted. In any case, our results are biochemically relevant and of potential pharmacological interest.

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MMP-9 activity (%)</th>
<th>MMP-2 activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080</td>
<td>47 ± 3</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>U2-OS</td>
<td>62 ± 1</td>
<td>27 ± 1</td>
</tr>
</tbody>
</table>

Experiments were carried out as described in Materials and methods in the absence or presence of 5 mM homocysteine. Data are given as percentages as compared to control values and they are means ± SD from two different, independent gelatin zymography assays.

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**Fig. 2** Effects of thiols and NADH added to the substrate buffer of the gelatin zymography assay. Conditioned media from control, untreated U2-OS cells were used for gelatin zymography. After electrophoresis, 5 mM of one of the tested thiols or NADH was added to substrate buffer.

**Fig. 3** Effects of homocysteine on the secretion of gelatinases by HT-1080 and U2-OS cells. Conditioned media from control and 5 mM homocysteine-treated cells were used for gelatin zymography as described in Materials and methods.
G. Bessede, C. Miguet, P. Gambert, D. Neel, G. Lizard, Efficiency


