An in vitro evaluation of the effects of homocysteine thiolactone on key steps of angiogenesis and tumor invasion

Beatriz Martínez-Poveda, Teresa Chavarría, Francisca Sánchez-Jiménez, Ana R. Quesada, and Miguel Ángel Medina*

Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of Málaga, E-29071 Málaga, Spain
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Abstract

Homocysteine thiolactone is a highly reactive homocysteine derivative that can react easily with proteins. Protein homocysteinylation has been suggested as a possible mechanism underlying the pathological consequences of impaired homocysteine metabolism. Homocysteine inhibits key steps of angiogenesis and tumor invasion. It can be hypothesized that homocysteine thiolactone could mimic the described anti-angiogenic and anti-invasive effects of homocysteine. Therefore, we studied the effects of homocysteine thiolactone on different key steps of angiogenesis and tumor invasion, using model endothelial and tumor cell lines. This study demonstrates that homocysteine thiolactone, in high contrast to homocysteine, is not an anti-angiogenic compound. Furthermore, our results suggest that homocysteine thiolactone could behave as a pro-angiogenic compound.

Keywords: Homocysteine thiolactone; Homocysteine; Angiogenesis; Bovine aorta endothelial cells; HT-1080 fibrosarcoma cells

In the last decade, homocysteine has attracted great interest because of its claimed involvement in cardiovascular disease as an independent risk factor [1]. In the last few years, this claim has raised strong discussion [2,3]. In fact, the most recent and complete published meta-analysis concludes that elevated homocysteine is at most only a modest independent predictor of ischemic heart disease and stroke risk [4]. However, this increased interest in homocysteine research has opened new previously unexplored ways, some of them not related at all with cardiovascular disease. Thus, impairment of homocysteine metabolism has been associated with folate or cobalamin deficiencies, pregnancy complications, neural tube defects, mental and cognitive disorders, and psoriasis, among others [3,5]. This has also been the case of previously published work in the potential modulatory role of homocysteine on angiogenesis [6–8] and tumor invasion [9,10]. In spite of all this evidence for a role of homocysteine in pathogenesis, mechanisms by which homocysteine can be harmful are largely unknown. In humans, homocysteine is involved in folate cycle and activated methyl cycle, as well as in transsulfuration reactions [3]. As a result of impairment in its metabolism, homocysteine can accumulate and becomes toxic to human cells, possibly due to its indirect incorporation into protein by methionyl-tRNA synthetase-mediated mechanisms involving homocysteine thiolactone (HTL) [11–13]. HTL is highly reactive and can react easily with proteins [13]. This protein homocysteinylation has been suggested as a possible mechanism underlying the pathological consequences of elevated homocysteine levels [14]. In fact, very recently, protein homocysteinylation has been demonstrated to occur in humans [15].

According to the previously mentioned relevant experimental background, we established the following working hypothesis: HTL could mimic the described anti-angiogenic and anti-invasive effects of homocysteine. To test this hypothesis, the present communication fulfills two main goals: a characterization of the effects of HTL on different key steps of angiogenesis in model...
cell culture systems and a comparative study of the effects of HTL on endothelial and tumor cells.

Materials and methods

Cell culture. Bovine aorta endothelial cells (BAEC), human HT-1080 fibrosarcoma, and U2-OS osteosarcoma cells were used. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (1 g/L for BAEC and 4.5 g/L for HT-1080 cells) or McCoy’s 5a medium (U2-OS cells). All media contained glutamine (2 mM) and they were supplemented with the antibiotics penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphotericin (1.25 μg/mL) and with 10% fetal bovine serum.

In HTL treatments of cells, culture media devoid of penicillin/streptomycin were used, since degradation of HTL by streptomycin has been previously described [12]. HTL was solved in culture medium, pH was re-adjusted since HTL acidifies the medium, and the solution was sterilized by filtering. HTL solutions were freshly prepared immediately prior to their use.

Proliferation assay. As much as 2.5 × 10⁴ cells in a total volume of 625 μL of medium were incubated in each well of 24-well plates with serial dilutions of HTL. After 3 days of incubation (37°C, 5% CO₂ in a humid atmosphere), cells were detached and counted by using a Coulter counter.

Endothelial cell differentiation assay: tube formation on Matrigel. Matrigel (50 μL of about 10.5 mg/mL) at 4°C was used to coat each well of a 96-well plate and allowed to polymerize at 37°C for a minimum of 30 min. Some 5 × 10⁴ BAE cells were added with 200 μL DMEM. Finally, different amounts of HTL were added and incubated at 37°C in a humidified chamber with 5% CO₂. After incubation for 7 or 20 h, cultures were observed (200× magnification) and photographed with a Nikon inverted microscope DIAPHOT-TMD (Nikon, Tokyo, Japan). Each concentration was tested in duplicate and two different observers evaluated the results of tube formation inhibition.

In vitro fluorogenic assay of MMP-9 activity. To carry out in vitro assays of MMP-9 activity, we purified this enzyme from conditioned media from PMA-treated HT-1080 cells, by affinity chromatography to gelatin A–Sepharose 4B [10]. Prior to their use for the in vitro assay of activity, samples of purified MMP-9 were completely pre-activated by treatment with 1 mM of 4-aminophenylmercuric acetate (APMA) for 12 h at 37°C. We used a fluorogenic assay with a peptide substrate highly specific for this gelatinase, as previously described [10].

Conditioned media and gelatinolytic assays. Conditioned media were prepared and gelatin zymography was carried out as previously described [9,10].

Migration and invasion assays. Migration and invasion of fluorescence-labeled tumor cells were assayed by using a 24-well fluorescence-opaque membrane insert. These assays allow us to monitor the process in real time, since they eliminate the need to remove nonmigratory cells before quantifying migratory cells. We have previously described the procedures elsewhere [9].

Results and discussion

Submillimolar concentrations of homocysteine thiolactone do not decrease endothelial and tumor cell proliferation rates

Cell proliferation is required in both angiogenesis and tumor progression and invasion. To determine whether treatments induced changes in the proliferation rates of endothelial and tumor cells, we treated BAEC and HT-1080 cells with different concentrations of HTL for 3 days, counted cells, and compared with cell counts in control, untreated cells. We found that only 5 mM homocysteine thiolactone produced a drastic decrease in cell count with obvious cytotoxic effect. On the other hand, 0.01–0.5 mM HTL did not decrease BAEC and HT-1080 cell proliferation rates (results not shown). These observations are in agreement with others previously reported on the lack of HTL toxicity for culture cells [16] and mouse embryos in vitro [17], on the support of cell growth by HTL in cultures in the absence of methionine [18], and on the lack of effects on endothelial and tumor cell viability [19,20]. However, Mercier et al. [19] reported that, in spite of the fact that human umbilical vein endothelial cell viability remained >95% for HTL concentrations ranging from 0.01 to 0.2 mM after 24–72 h of incubation, 0.05 and 0.2 mM HTL induced caspase-independent apoptosis in a significant percentage of cells. In the same direction, Huang et al. [20] have recently shown that HTL can induce apoptosis in HL-60 cells, although only at 0.5 mM or higher concentrations of HTL. Since we only carried out cell counts, we cannot rule out that part of the viable cells could be in initial phases of the apoptotic process. This is not a fully closed issue, since other reports show that HTL can partially inhibit apoptosis induced by 3-deazaadesonine onHL-60 cells [21]. Further experimental effort would be needed to study the cell-type specificity of the pro- and anti-apoptotic effects of HTL.

Homocysteine thiolactone has no effect on endothelial cell differentiation

At the final phase of angiogenesis, endothelial cells halt their proliferation and form a new vessel. Fig. 1

Fig. 1. Homocysteine thiolactone has no relevant effect on the endothelial cell differentiation assay. This assay was carried out as described under Material and methods using BAEC cells, either in the absence (A) or the presence of 0.1 mM (B) or 5 mM (C) homocysteine thiolactone.
shows that homocysteine thiolactone has no relevant effect on endothelial cell differentiation on Matrigel. These results are in contrast with those showing an inhibitory effect of homocysteine on endothelial cell differentiation [7]. On the other hand, we have previously observed that, under certain culture conditions (high cell density, absence of serum, and treatment with 5 mM homocysteine for 4–6 h), homocysteine can induce the formation of structures on culture dishes similar to those obtained in the differentiation assay on Matrigel [8]. We could not reproduce this effect with homocysteine thiolactone treatment (results not shown). In any case, homocysteine thiolactone cannot mimic the effects of homocysteine on endothelial cell differentiation.

Homocysteine thiolactone partially inhibits gelatinase activity

Since both angiogenesis and tumor invasion and metastasis require remodeling of extracellular matrix, and gelatinases (MMP-2 and 9) are mainly involved in this remodeling [22,23], we investigated whether homocysteine thiolactone can inhibit both activities.

Conditioned media from phorbol ester-treated human fibrosarcoma HT-1080 cells are a very good source of MMP-9 protein. However, in these conditioned media MMP-2 protein is also present [24], though at lower levels. Thus, a previous purification of MMP-9 was required in order to carry out the in vitro assays of MMP-9 activity. In our hands, the purification procedure described in the experimental section yielded a homogeneous, very active MMP-9 suspension.

Fig. 2 shows that homocysteine thiolactone can inhibit, but only partially, MMP-9 activity. In fact, at a concentration as high as 1 mM, homocysteine thiolactone could inhibit MMP-9 activity by only 50%, and even at 5 mM homocysteine thiolactone 15% of total MMP-9 activity remained. These data are in high contrast with previous observations on the inhibitory effect of homocysteine and other thiols on gelatinase activity [8,25,26]. Furthermore, we have found that 75 μM homocysteine completely inhibits MMP-9 activity [10]. Thus, homocysteine thiolactone behaves as a much milder inhibitor of gelatinases than homocysteine and other thiols.

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 [24]. To show whether this inhibitory effect of homocysteine thiolactone 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 (see Fig. 3). In this assay, partial inhibition of both gelatinases is shown for 0.5 mM homocysteine thiolactone and only a weak band of gelatinase activity was detectable when 2 mM homocysteine thiolactone was added to the substrate buffer. Since exposition of the gels to high concentrations of HTL can produce homocysteinylation of gelatin, the observed effect of 2 mM HTL could be— at least, partly— due to a putative resistance of modified gelatin to gelatinase activity.

Conditioned media from both tumor cell lines exposed for 24 h to different concentrations of homocysteine thiolactone (0.001–0.5 mM) showed no relevant inhibition of both MMP-2 and MMP-9, as determined by gelatin zymography (Fig. 4).

All the available data seem to indicate that the inhibitory effect of homocysteine on gelatinases is due to its reactive sulphydryl group and that, in fact, homocysteine is the physiological thiol with the most potent inhibitory effect on MMP-9. Since homocysteine thiolactone has the thiol group blocked, its inhibitory effect on gelatinases should be due to a different mechanism, maybe related to protein homocysteinylation.
conclusion, the effects of homocysteine and homocysteine thiolactone on gelatinases are neither quantitative nor mechanistically comparable.

Homocysteine thiolactone stimulates tumor cell migration and has no relevant effect in the invasion assay

In our recent report on the anti-angiogenic effects of homocysteine on cultured bovine aorta endothelial cells, we have shown that the presence of 5 mM homocysteine produces a marked inhibition of both invasion and migration [8]. These data supported the previous observation by Nagai et al. [7] of an inhibitory effect of homocysteine on bovine aorta endothelial cells. Since invasive potential is a common feature in the processes of angiogenesis, tumor progression, and metastasis [22,23], a similar effect on tumor cells is expected. In fact, we have recently shown that homocysteine inhibits both migration and invasion of HT-1080 cells [9]. Similar effects have been shown for glutathione, other main circulating low molecular weight thiol, on malignant tumor cell invasion [25]. The other thiol, N-acetyl-cysteine, has also been shown to inhibit the invasion of a reconstituted basement membrane by both malignant tumor cells and proliferative endothelial cells [25,26].

Fig. 5 shows the effect of 0.5 and 5 mM homocysteine thiolactone on HT-1080 migration and invasion. No cytotoxic effect was observed during the assay time with those concentrations of homocysteine thiolactone. In complete contrast with the inhibitory effect of homocysteine, we show a dramatic stimulatory effect of both concentrations of homocysteine thiolactone on HT-1080 migration (see Fig. 5A). On the other hand, tumor cell invasion was only slightly inhibited at the highest concentration tested (see Fig. 5B). Therefore, the effects of homocysteine thiolactone on cell migration and invasion are completely different to those previously shown for homocysteine and other thiols.

Concluding remarks

Based on previous results on the effects of homocysteine, we formulated the hypothesis of a potential anti-angiogenic and anti-invasive role of its highly reactive derivative, homocysteine thiolactone. In the present work, we have tested our working hypothesis. Our results leave no doubt: the working hypothesis should be rejected. In high contrast with the previously published anti-angiogenic and anti-invasive effects of homocyste-
ine [6–10], herein we have shown that homocysteine thiolactone might behave rather as a pro-angiogenic and pro-invasive compound. Future experimental work should elucidate the actual physiopathological relevance of these new data.

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