Endothelin-1 promotes proteolytic activity of ovarian carcinoma

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ABSTRACT

Endothelin-1 (ET-1) is a potent mitogenic and angiogenic factor for ovarian carcinoma cell lines, which acts selectively through the ET<sub>A</sub> receptor (ET<sub>A</sub>R). A previous study demonstrated that ET-1 is present at high concentrations in ovarian cancer ascites, indicating a direct role in the progression and metastasis of ovarian carcinoma. In this study, we investigated whether ET-1 could induce production and activation of tumour-associated proteinases in ovarian carcinoma cells. As demonstrated by ELISA, we found that the secretion of matrix metalloproteinase (MMP)-2 and MMP-9, urokinase-type plasminogen activator and plasminogen activator inhibitor type-1 and -2 was upregulated by ET-1 in a dose-dependent manner in the HEY cell line. In addition, the MMPs in ET-1-treated cells are consistently active, as shown by MMP gelatinase activity assay. Finally, we demonstrated that BQ-123, an antagonist of ET<sub>A</sub>R, inhibited the ET-1-induced tumour protease secretion and activity, suggesting that ET-1/ET<sub>A</sub>R may play an important role in the progression and metastasis of ovarian carcinoma, activating multiple protease cascades.

INTRODUCTION

Ovarian cancer has one of the highest mortality rates among the gynaecological malignancies [1]. Initiation of invasive and metastatic cascades is triggered by degradation of basement membrane components by specific proteinases. Endothelin-1 (ET-1) is a peptide produced primarily by vascular cells and in elevated amounts by different cancer cells [2]. We have demonstrated previously that ET-1 is overexpressed in primary and metastatic carcinoma compared with normal ovarian tissues, and in ovarian tumour cells ET-1 acts as an autocrine growth factor via the ET<sub>A</sub> receptor (ET<sub>A</sub>R) [3–5]. Our previous findings demonstrated that in ascitic fluids of patients with ovarian carcinoma ET-1 and vascular endothelial growth factor levels are elevated [6]. The presence of high levels of ET-1 in ascites indicates that this peptide could participate in the progression and invasion of ovarian carcinoma. Moreover, we demonstrated that among the angiogenic responses in cultured endothelial cells, including proliferation, migration, invasion and morphogenesis, ET-1 stimulated mRNA and protein expression of matrix metalloproteinase (MMP)-2, the major secreted proteolytic enzyme of the extracellular matrix (ECM) [7]. All these findings suggested that ET-1 could enhance the secretion of metastasis-associated proteinases and thereby facilitate cell invasion. The MMPs are a family of at least 18 secreted and membrane-bound zinc-endopeptidases [8]. Collectively, these enzymes can degrade all the components of the ECM.

Key words: endothelin A receptor, endothelin A receptor antagonist, invasion, matrix metalloproteinase, urokinase-type plasminogen activator.

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; ET-1, endothelin-1; ET<sub>A</sub>R, ET<sub>A</sub> receptor; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

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including fibrillar and non-fibrillar collagens, fibronectin, laminin and basement membrane glycoproteins. All MMPs are synthesized as pro-enzymes, and most are secreted from the cells in this form. Thus, the activation of these pro-enzymes is a critical step that leads to ECM breakdown. Numerous studies have shown that there is a close association between expression of various members of the MMP family by ovarian tumours and their invasive behaviour and metastatic potential [9,10]. Among the proteases that play an active role in the tumour progression are the serine proteases of the urokinase-type plasminogen activator (uPA) system. uPA is secreted as an inactive precursor (pro-uPA) that binds with high affinity to a specific cell surface receptor (uPAR) [11]. Concomitant binding of pro-uPA to uPAR and plasminogen strongly enhances plasmin generation. Moreover, plasmin is a potent activator of most pro-MMPs. In the present study we demonstrated for the first time the role of ET-1 in enhancing the proteolytic capability of ovarian cancer cells, suggesting a direct role in the biological invasiveness of human ovarian cancer.

MATERIALS AND METHODS

Cell culture
The human ovarian carcinoma cell line HEY was a gift from Dr G. Scambia (Catholic University School of Medicine, Rome, Italy) and was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal calf serum and 1% penicillin/streptomycin. All culture reagents were obtained from Gibco/BRL, Life Technologies (Paisley, U.K.). To perform experiments for analysis of proteinases, cells were plated in 100 mm Petri dishes.

Preparation of conditioned medium
Subconfluent cultures of HEY cells were washed three times with PBS and starved for 24 h in serum-free DMEM to reach quiescence. The medium was discarded and replaced with fresh serum-free DMEM containing ET-1 (Peninsula Laboratories, Belmont, CA, U.S.A.). The cells were then incubated for an additional 24 h. The conditioned medium was then collected, centrifuged and stored in aliquots at −20 °C. The remaining cells were counted and the data were corrected for the cell number.

Measurement of MMPs and uPA/plasminogen activator inhibitor 1 and 2 (PAI-1 and -2) protein by ELISA
Gelatinase activities in conditioned medium were determined using a MMP Gelatinase Activity Assay Kit (Chemicon International, Inc., Temecula, CA, U.S.A.) using the manufacturer’s instructions. The sensitivity of the assay is less than 5 ng/ml of MMP in a range of 10–200 ng/ml. MMP-2 levels in conditioned medium were measured using a BioTrak Human MMP-2 ELISA kit (Amersham, Arlington Heights, IL, U.S.A.), using the manufacturer’s instructions. MMP-2 can be detected in the range 1.5–24 ng/ml. There is no cross-reaction with MMP-1, -3, -7, -8, -9 and membrane type 1-MMP. MMP-9 levels in conditioned medium were measured using MMP-9 ELISA kit (Oncogene Research Products, Cambridge, MA, U.S.A.), using the manufacturer’s instructions. MMP-9 can be detected in the range 0.625–20 ng/ml. uPA, PAI-1 and PAI-2 were determined by using commercially available ELISA kits (American Diagnostica, Greenwich, CT, U.S.A.), following the manufacturer’s instructions. uPA, PAI-1 and PAI-2 can be measured in the ranges 0.1–1 ng/ml, 0.05–10 ng/ml and 0.05–10 ng/ml respectively.

RESULTS

Using human MMP ELISA kits, we measured the effect of exogenous ET-1 on the secretion of MMP-2 and MMP-9, by ovarian carcinoma cell line HEY, which expresses abundant high-affinity receptor for ET-1 (Kd = 0.1 nM; 36500 receptors/cell). As shown in Table 1, ET-1 enhanced MMP-2 and MMP-9 expression in a dose-dependent manner. The difference in MMP-2 and MMP-9 secretion between stimulated and unstimulated cells was significant at all of the ET-1 concentrations tested. A selective ET_A R antagonist, BQ-123, was used to determine whether the ET_A R was involved in the stimulation of MMP-2 and MMP-9 secretion in ovarian carcinoma cells. ET-1-stimulated MMP-2 and MMP-9 secretion was completely blocked by the addition of 1 μM BQ-123. Evaluation of MMP activity using a MMP gelatinase activity kit demonstrated the net increase in MMP activity, showing a 2.5-fold increase in ET-1-treated cultures. The addition of 1 μM BQ-123 completely blocked the MMP activity, suggesting that ET-1 was able to enhance secretion and activation of MMP-2 and MMP-9 in ovarian carcinoma cells through ET_A R.

uPA and PAIs have been proposed to play a role in ovarian carcinoma cell invasion, and high levels have been correlated with the progression and metastasis of ovarian cancer [12]. The ET-1-induced stimulation of uPA and PAI-1/-2 secretion by HEY cells was measured using ELISA kits. This effect was dose-dependent in the range between 0.1 nM and 100 nM, with a maximum increase (approx. 2.5-fold) observed at a concentration of ET-1 of 100 nM (Table 1). Pretreatment of cells with the ET_A R antagonist, BQ-123, prevented the stimulation of uPA and PAI-1/-2 secretion induced by ET-1, confirming that the ET_A R subtype mediates the stimulation of uPA system.
Table 1  Summary of effects of ET-1 on the ovarian cancer cell line HEY
Serum-starved HEY cells were treated with different concentrations of ET-1 in serum-free medium for 24 h. Conditioned medium was assayed in duplicate and the results are the means from three different experiments. Concentrations are expressed as ng/ml; mean ± S.D. MMP activity was measured as relative activity with respect to control.

<table>
<thead>
<tr>
<th></th>
<th>[MMP-2]</th>
<th>[MMP-9]</th>
<th>MMP activity</th>
<th>[uPA]</th>
<th>[PAI-1]</th>
<th>[PAI-2]</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10.8 ± 2.0</td>
<td>5.37 ± 0.20</td>
<td>100</td>
<td>1.86 ± 0.20</td>
<td>25.4 ± 7.0</td>
<td>9.0 ± 1.6</td>
</tr>
<tr>
<td>ET-1 (100 nM)</td>
<td>23.7 ± 1.0</td>
<td>22.2 ± 1.00</td>
<td>272</td>
<td>6.0 ± 1.8</td>
<td>50 ± 12</td>
<td>26 ± 11</td>
</tr>
<tr>
<td>ET-1 (10 nM)</td>
<td>22.6 ± 1.0</td>
<td>17.3 ± 1.5</td>
<td>250</td>
<td>4.8 ± 1.3</td>
<td>30.4 ± 9.0</td>
<td>18.6 ± 9.6</td>
</tr>
<tr>
<td>ET-1 (1 nM)</td>
<td>16.3 ± 1.3</td>
<td>15.7 ± 1.3</td>
<td>219</td>
<td>3.2 ± 0.8</td>
<td>30 ± 7</td>
<td>18.0 ± 8.6</td>
</tr>
<tr>
<td>BQ-123 (1 μM)</td>
<td>9.0 ± 1.5</td>
<td>4.8 ± 1.7</td>
<td>120</td>
<td>1.66 ± 0.90</td>
<td>22 ± 4</td>
<td>6.8 ± 2.0</td>
</tr>
<tr>
<td>BQ-123 (1 μM) +</td>
<td>11.6 ± 1.8</td>
<td>7.0 ± 1.6</td>
<td>130</td>
<td>1.94 ± 0.60</td>
<td>23.2 ± 2.8</td>
<td>10.5 ± 2.1</td>
</tr>
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</table>

DISCUSSION

Tumour progression of ovarian cancer involves the disruption of anatomical barriers and penetration of tumour cells into normal adjacent tissues. Such migratory and invasive events are regulated by different proteolytic systems [13]. Several growth factors that bind to tyrosine kinase receptors or to G-protein-coupled receptors act as positive regulators of tumour invasion, stimulating tumour-associated proteinases and tumour cell motility [14–16].

Our previous results indicated that the tumour-promoting activity of ET-1 occurs through an autocrine pathway that stimulates ovarian carcinoma cell proliferation through ET₄R, which is predominantly expressed in tumour cells. The presence of high ET-1 levels in ascites of patients with ovarian carcinoma implies that this molecule is relevant in the progression and invasion of ovarian carcinoma. Numerous studies have documented the important role of gelatinases/type IV collagenases (MMP-2 and MMP-9) in ovarian tumour invasion, suggesting a new functional role in supporting tumour growth, modulating ECM, regulating the availability of growth factors and facilitating the angiogenesis [17]. In this paper we have shown that ET-1 is able to stimulate an increased production and activation of MMP-2 and MMP-9. The ability of ET-1 to enhance the enzymic activity of MMP-2 and MMP-9 through the activation of their latent forms indicates an important role of ET-1 in the invasive characteristics of ovarian tumour cells. It is generally believed that uPA at the cell surface initiates a proteinase cascade, which in turn leads to breakdown of the ECM and thereby promotes cellular invasion [18]. This conclusion is supported by the fact that uPA is highly expressed by tumour cells, and in ovarian carcinoma they are independent prognostic indicators [19]. Our results indicate the ability of ET-1 to enhance the secretion of uPA in ovarian cancer cells, contributing to their invasiveness. Fibrinolysis by plasmin is controlled by several inhibitors, such as PAIs. Nevertheless the results of studies of the significance of PAIs in various cancers have been conflicting. Recent studies indicate that PAI-1 acts as a cell-detachment factor, explaining, in part, the correlation between its

Figure 1  ET-1 enhances proteolytic activity of ovarian carcinoma cells
ET-1 modulates the co-operation between MMPs and serine proteinases for enzymic activation involved in ECM degradation, tumour cell invasion and metastasis.
increased levels and poor prognosis, and the reduced tumour invasion and vascularization observed in PAI-1 deficient mice [20]. The results presented here demonstrate that ET-1 stimulates PAI-1 and PAI-2 secretion in ovarian carcinoma cells, leading to increased degradation of ECM, which might lead to tumour growth and metastasis.

Recent studies focused on the different roles of proteinases demonstrate the co-operation between MMPs and uPA. It is likely that some of these proteinases serve redundant functions, so that activation of multiple pathways might be required to elevate proteolytic activity [21]. Some MMPs, such as MMP-3, are activated intracellularly by serine proteinases. Thereafter, in concert, plasmin and MMP-3 activate other MMPs (MMP-13, MMP-9 and MMP-7), resulting in a cascade of activation. Our results indicate that the ET-1-dependent activation of MMPs may have involved a uPA/plasminogen-independent proteolytic processing system. On the other hand, our studies have shown that ET-1 increased uPA system secretion in ovarian carcinoma cell lines, suggesting a parallel co-regulation of enzymic activities of MMPs. The interactions between different enzymic systems and the action of ET-1 on their secretion and activation represents one possible mechanism linking ET-1 to ovarian progression and metastasis (Figure 1).

In this study, we identified ET-1 as a key component of tumour progression and invasion by acting as tumour protease activity regulator. The autocrine production of ET-1 by ovarian cancer cells and the selective binding to ET1R expressed on ovarian cancer cells, lead to an increased potential for tumour cell proliferation and cellular invasive activity. Thus, the therapeutic use of specific ET1 receptor antagonists may provide an additional approach to the treatment of ovarian carcinoma through ET1 receptor blockade, which may result in the reduction of tumour growth and invasion.

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