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Influence of IGF-I on adhesion, proliferation, and galectin-1 production in JAr and Jeg-3 choriocarcinoma cell lines

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ABSTRACT

BACKGROUND: *JAr and Jeg-3 choriocarcinoma cell lines are model systems for the transformed trophoblast and allow studies of phenotype and regulatory factors for particular cell functions. Both cell lines express the receptor for insulin-like growth factor-I (IGF-I). Effects of IGF-I on adhesion, proliferation and galectin-1 production in JAr and Jeg-3 cells were studied.*

METHODS: *The effects of IGF-I on proliferation and galectin-1 production were examined by thiazolyl blue assay and cell based solid phase assay using polyclonal anti-galectin-1 antibodies. The cell adhesion assay was performed on Matrigel coated wells. Galectin-1 production and localization was examined by immunocytochemistry.*

RESULTS: *IGF-I decreased adhesion of JAr cells to 70% of the control value ($p<0.05$). Cell treatment with 10 μg/L of IGF-I significantly increased viable cell number: by 13.5% in JAr and 6% in Jeg-3. Gal-1 was immunolocalized intracellularly and associated with the cell membrane in both cell lines. Production of galectin-1 was significantly increased after treatment with IGF-I compared to control: by 7% in JAr cells and by 16% in Jeg-3 cells ($p<0.05$).*

CONCLUSION: *The data showed that IGF-I affected adhesion and proliferation of choriocarcinoma cells, depending on the cell line. Both choriocarcinoma cell lines studied here produced galectin-1. The amount of galectin-1 was moderately stimulated by IGF-I.*

KEY WORDS: *Cell Line, Tumor; Choriocarcinoma; Insulin-Like Growth Factor I; Galectin 1; Immunohistochemistry*

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Abbreviations:

IGF-I - Insulin-like Growth Factor-I, MTT - thiazolyl blue, CELISA - Cell based solid phase assay, gal-1 - galectin-1, RT - Room Temperature

INTRODUCTION

Choriocarcinoma, originating from the trophoblast of the human placenta, is a relatively rare malignant tumor (1). A number of choriocarcinoma cell lines was established, among them BeWo, JAr, and Jeg-3 cell are the most widely used as model systems. All three cell lines secrete the major trophoblast hormone hCG, but they differ in their morphology, proliferative activity and degree of differentiation. JAr spheroids maintain their cytotrophoblast like morphology, while BeWo and Jeg-3 spheroids expose flattened and more differentiated cells on their surfaces (2). However, these cell lines have an invasive phenotype, proliferate (2,3), and express receptor for the insulin-like growth factor-I (IGF-I) (4). The IGF system plays an important role in normal growth and development, and in a variety of pathological situations, particularly tumorigenesis (5). There is epidemiological evidence that high levels of circulating IGF-I constitute a risk factor for the development and progression of human epithelial cancers such as breast, prostate and colon cancer (5). IGF-I is also a potent mitogen in cell culture (5). Treatment with IGF-I *in vitro*, strongly inhibited apoptosis in colon carcinoma cells (6), prostatic cancer cells (7), and MCF7 breast cancer cells (8).

There are clinical and experimental evidences, which support involvement of galectin-1 (gal-1) in cancer development (9). This lectin, with affinity for β-galactoside residues (10), has specific ligands identified as extracellular matrix (ECM) proteins, laminin and fibronectin (11,12). Gal-1 is involved in several biological events, including cell-cell and cell-matrix interactions (13). It has been demonstrated that the expression pattern of gal-1 is altered in carcinomas, including those arising from thyroid, endometrium, head and neck, thymus, bladder, pancreas, colon, ovary (14), and trophoblast (15). Many transformed cell lines of different origin also express gal-1 (16-18). However, not much is known regarding regulation of gal-1 expression, in neither normal nor the transformed cells. Therefore, the aim of this study was to investigate the possible effects of IGF-I on adhesion and proliferation of JAr and Jeg-3 choriocarcinoma cells and their production of gal-1.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) with and without phenol red, Minimum Essential Medium Eagle's (MEM Eagle's) without phenol red, antibiotic/antimycotic mixture (100 μg/mL streptomycin sulfate, 100 units/mL penicillin G and 25 μg/mL amphotericin B), thiazolyl blue (MTT), bovine serum albumin (BSA) and insulin-like growth factor-I (IGF-I) were from Sigma (Chemical Co., USA). Fetal calf serum (FCS) was obtained from PAA Laboratories (Linz, Austria). MEM Eagle's culture media with phenol red was from the Institute of Immunology and Virology (Belgrade, Serbia and Montenegro). Horseradish peroxidase (HRP) and 3,3',5,5'- tetramethyl benzidine (TMB) were from ICN Biochemicals (Aurora, Ohio, USA). Growth factor reduced Matrigel matrix was obtained from BD Biosciences (Becton Dickinson, USA). Vectastain Elite ABC kit and

3,3'-diaminobenzidine (DAB) kit were from Vector Laboratories (Burlingame, CA, USA). Laboratory plasticware were from Falcon (Becton Dickinson, USA) and Costar (Corning Incorporated, NY, USA). Other chemicals were p.a. grade.

Antibodies

Antiserum to human placental gal-1 was produced in our laboratory as described earlier (16,19). It showed no cross-reactivity with other placental galectins as previously documented by Western blot analysis. Sheep anti-rabbit IgG-HRP conjugate was produced in our laboratory according to the method of Nakane (20).

Cell culture

Jeg-3 choriocarcinoma cells from the European Collection of Cell Cultures (ECACC, Salisbury, UK, passage number 151) were cultured in MEM Eagle's media, containing 10% FCS and antibiotic/antimycotic mixture. JAr choriocarcinoma cells (ATCC, USA, donated by Dr Sue Greenwood) were cultured in DMEM/F12 supplemented with 10% FCS and antibiotic/antimycotic mixture. Cells were grown in tissue culture flasks for propagation, in 96 well plates for CELISA and adhesion assays, or on glass coverslips for immunocytochemistry.

Immunocytochemistry

Jeg-3 and JAr choriocarcinoma cell lines ($2x10^5$) were cultured on glass coverslips in the respective media. The cells were rinsed with phosphate buffered saline (PBS 0.05 M, pH 7.2), air dried and fixed in 4% paraformaldehyde (30 min.). Endogenous peroxidase activity was blocked with 0.3% H_2O_2. After blocking of nonspecific binding (Vectastain Elite ABC kit) for 20 min., cells were incubated with anti-gal-1 antiserum (1/4000 dilution) for 2 hours at room temperature (RT) and stained using the Vector ABC kit with DAB as chromogen. Slides were then dehydrated, mounted and examined under Reichert-Jung microscope with Photostar automatic camera system (Vienna, Austria). Negative controls with PBS or nonimmune rabbit IgG (Vector, CA, USA) in place of the anti-galectin-1 antiserum were performed routinely and resulted in the complete absence of staining.

Treatment with IGF-I

The effect of IGF-I was studied in three independent experiments using media containing FCS. Jeg-3 and JAr cells were seeded in 96-well plates at $2x10^4$cells/well and cultured in humidified 5% CO_2/95% air at 37ºC. The medium was removed after 24 hours and cells were rinsed once with PBS. Cells were incubated in the respective media with IGF-I at final concentrations of 10 (n=15) and 20 μg/L (n=15) and without IGF-I (control group, n=20). After 48 hours, confluent cells were washed once with PBS and fixed with ice-cold methanol/acetone (1:1) at RT for 10 min., dried and stored at -20ºC until use in cell based ELISA.

Cell based ELISA (CELISA)

JAr and Jeg-3 cell monolayers were rehydrated with PBS (3x5min) and blocked with 1% BSA (200 μL/well) for 1 hour at RT. Rabbit anti-galectin-1, 50 μL/well (0.3 mg/mL IgG in PBS, with 1% BSA) was incubated for 2 hours at RT. The plates were then washed (3 x 5 min.) with PBS-0.05% Tween-20 (PT). Sheep anti-rabbit-IgG-HRP 50 μL/well (1/1000 in PBS-1% BSA) was incubated for 2 hours at RT. Wells were washed (5 x 5 min.) with PT and incubated with 50 μL/well of substrate (0.003% H_2O_2) and 50 μL/well of chromogen (0.05% TMB). The reaction was stopped with 0.2 M H_2SO_4 (100 μL/well). Absorbance was measured at 450 nm using Micro plate reader (LKB, 5060-006) and normalized to cell number. For assessment of non-specific binding, nonimmune rabbit IgG was used in place of anti-galectin-1.

Determination of viable cell number

For cell quantitation, JAr and Jeg-3 ($2x10^4$cells/well) were plated in 96-well plates and incubated with IGF-I (10μg/L, n=6) and without treatment (n=12), as described above. After treatment, 100 μL of thiazolyl blue (MTT, 1 mg/mL in DMEM/F12 and MEM Eagle's without phenol red) was added to each well. After incubation for 2 hours at 37ºC, the medium was replaced by 1-propanol (100 μL/well) and the plates were vigorously shaken to ensure complete solubilization of the blue formazan (21). Absorbances was measured at 570 nm and compared to the standard curve obtained with $5x10^3$, $1x10^4$, $2x10^4$, $4x10^4$, $6x10^4$, and $8x10^4$ cells/well.

Adhesion assay

Adhesion assay was a modification of a previously described procedure (22). 96-well plates were precoated with 250 μg/mL of Matrigel for 1 hour at 37ºC, treated with 50 μL of 3% BSA to block non-specific binding sites, washed with PBS and used in adhesion assay. The trypsinized JAr and Jeg-3 cells were resuspended at a final concentrations of $2.5x10^5$ cells/mL in the respective serum free media with 0.1% BSA. Cells were preincubated with 10 μg/L of IGF-I (n=6) or without IGF-I (control group, n=6) for 1 h in a humidified 5% CO_2/95% air at 37ºC with occasional agitation and plated in precoated 96 wells at $2.5x10^4$ cells/well. After incubation for 1 h, the plates were rinsed once gently with PBS. The attached cells were stained using 50 μL of 0.4% crystal violet in 10% ethanol and 1% ammonium oxalate for 5 min. The excess of dye was removed by immersing the plates in water and dried at RT. The incorporated crystal violet was dissolved in 100 μL/well of 33% glacial acetic acid and optical density was read at 540 nm. The intensity of staining was proportional to the number of the adhered cells.

Data analysis

Values expressed as percent of control are given as mean ± SD. The one-way ANOVA was applied for statistical analysis, with statistical significance of $p<0.05$.

RESULTS

Adhesion

Figure 1A illustrates the effect of IGF-I (n=6) on adhesion of JAr and Jeg-3 cells. Adhesion of JAr to Matrigel was inhibited by 30% ($p<0.05$), compared to the control (n=6). No effect, however, was observed in adhesion of Jeg-3 cells to Matrigel under the same experimental conditions.

Viable cell number

Treatment of JAr and Jeg-3 cells with IGF-I was found to influence the cell number, as documented by the MTT assay (Figure 1B). The viable cell number of JAr cells increased by 13.5% when the cells were cultured in medium containing 10 μg/L of IGF-I (n= 6, $p<0.05$), compared to the control (n=12). The number of Jeg-3 cells was increased by 6% after treatment with 10 μg/L of IGF-I (n=6) compared to the control levels (n=12, $p<0.05$). Treatment with 20 μg/L of IGF-I was less effective in both cell lines (data not shown).

Gal-1 production

The result for gal-1 production in both cell lines normalized for the IGF-I effect on cell number, is shown in Figure 1C.

Compared to the control (n=60), treatment of JAr cells with 10 μg/L of IGF-I increased gal-1 production by 7% (n=45, $p<0.05$) and in Jeg-3 cells by 16% (n=45, $p<0.05$). Concentration of 20 μg/L of IGF-I had no significant effect on gal-1 production in either cell lines (data not shown).

Immunolocalization of gal-1 in JAr and Jeg-3 cells

Immunostaining for gal-1 in JAr and Jeg-3 cell lines is shown in Figures 2A and 2B. Gal-1 was localized in the cytoplasm and perinuclearly (Figure 2, A and B, arrowheads), but also associated with the cell membrane and extracellular matrix (Figure 2, A and B, arrows). Treatment with 10 μg/L of IGF-I did not result in microscopically evident differences in immunolocalization (data not shown).

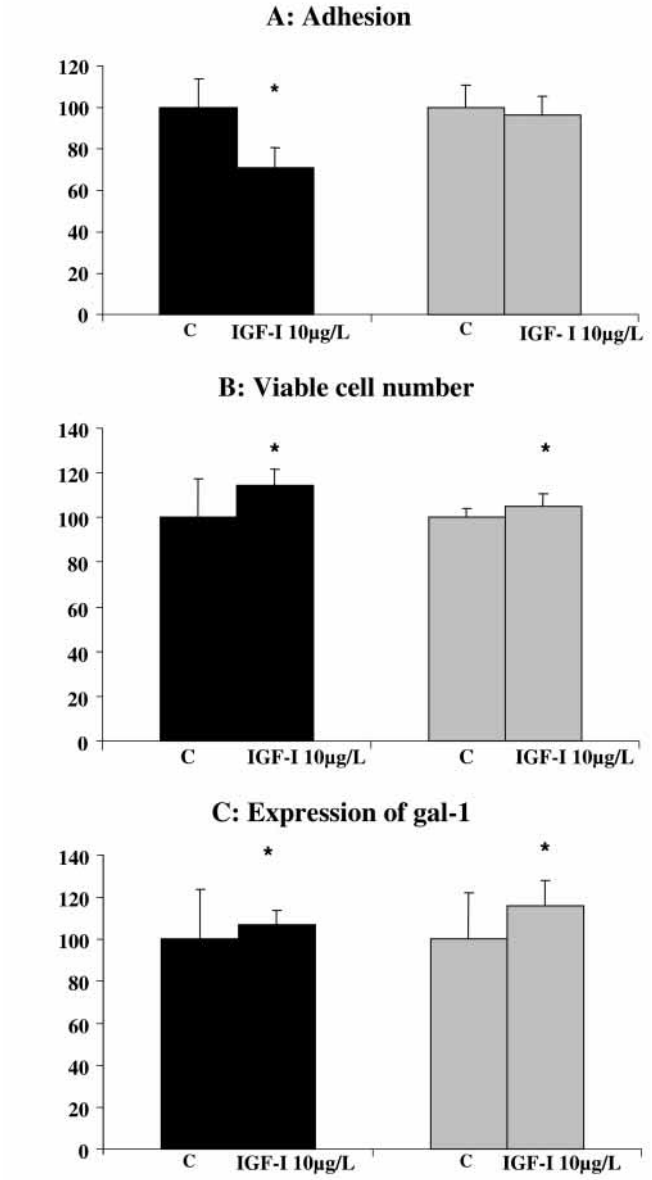

Figure 1. Effect of IGF-I (10 µg/L) on choriocarcinoma cells

A) Adhesion of JAr (black bars) and Jeg-3 (gray bars) to Matrigel after treatment with IGF-I. Data for the treated cells are expressed as percent of control and represented by the mean ±SD (significant at $p<0.05$, *); n=6 for both cell lines, in control (C) and the treatment group.

B) Viable cell number of JAr (black bars) and Jeg-3 (gray bars) cell lines, after treatment with IGF-I. Data are expressed as percent of control and represented by the mean ±SD (significant at $p<0.05$, *). Control, for both cell lines n=12; treatment group n=6.

C) Galectin-1 production by JAr (black bars) and Jeg-3 (gray bars) choriocarcinoma cell lines treated with IGF-I. Data are normalized for the cell number (mean ±SD) and expressed as percent of control (significant at $p<0.05$,*). The presented data are from three independent experiments (n=60 in control, and n=45 in treatment group).

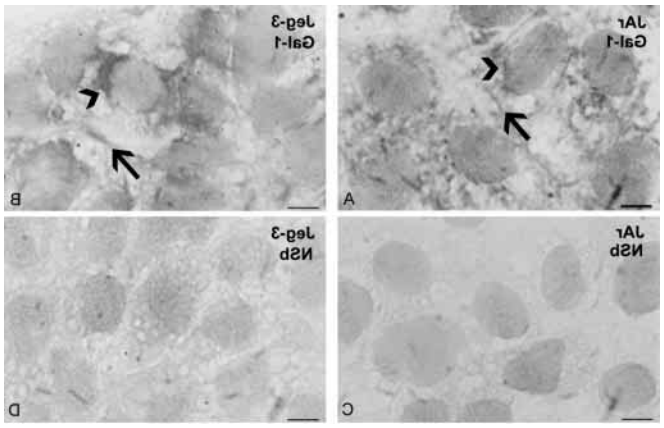

Figure 2. Immunolocalization of galectin-1 in JAr (A) and Jeg-3 (B) choriocarcinoma cell lines; JAr cells (A) and Jeg-3 cells (B) express galectin-1 intracellularly at perinuclear location (arrowheads). In both cell lines, galectin-1 is also membrane-associated (arrow). Negative controls (NSb) for JAr (C) and Jeg-3 (D) cells show complete absence of staining. Cells were counterstained with hematoxylin. Scale bar 10μm

DISCUSSION

This study aimed to investigate the effects of IGF-I on cell adhesion and proliferation, and gal-1 production in JAr and Jeg-3 choriocarcinoma cell lines. In this study, it is demonstrated that IGF-I inhibited adhesion of JAr cells to Matrigel, while adhesion of Jeg-3 cells was not affected. Other authors have reported similar decrease in cell adhesion using human peripheral blood monocytes with IGF-I concentrations 100-fold higher (23).

In normal trophoblast IGF-I increased adhesion to fibronectin (24), but the transformed trophoblast has not been studied so far. IGF-I is a mitogen for many transformed cells in vivo and *in vitro* (5,25,26). It is well known that IGF-I and signaling through IGF-I receptor, in general, stimulates DNA synthesis (27), cell growth (28), and protects cells from apoptosis (29). IGF-I affects proliferation and invasion of choriocarcinoma cells, reduces proliferation of JAr cells, and has no effect on Jeg-3 cell number, in the presence of FCS (30). Our study, however, showed that IGF-I, in the presence of FCS, moderately stimulated viable cell number in both JAr and Jeg-3 cell lines. The discrepancy in the findings of Mandl et al. (30) and our study may have resulted from differences in the stage of cell differentiation, as well as from different methods used for cell counting. The method used in our study is the direct one and counts only viable cells *in situ*, as opposed to the method of Mandl et al. (30) which counts particles obtained after detachment with enzymes that includes dead cells, present in considerable amounts in medium with FCS. Since the increase in viable cell number, shown in our study is rather modest, it is possible that it fell within the standard error of the method used by Mandl et al. (30).

Possible effect of IGF-I on cell differentiation was studied with respect to the gal-1 production, which is assumed to play a role in cell adhesion, migration and invasion. Cell adhesion, in general, is mediated by interaction between the matrix proteins and one or more cell-surface integrins (31). Gal-1 could modulate these interactions, positively or negatively (31). Previously, BeWo choricarcinoma cell line was shown to produce gal-1 (16). We are now extending this finding to show that JAr and Jeg-3 choriocarcinoma cells also produce this galectin. After treatment with IGF-I, no microscopically evident differences in anti-gal-1 staining were observed. The same effect was studied using CELISA, which however, showed that IGF-I treatment induced statistically significant differences in gal-1 production compared to the control in both cell lines. Since treatment with IGF-I at 10 μg/L was shown to affect cell numbers in both studied cell lines, the data for gal-1 production determined by CELISA were normalized to cell count for the observed increase. Both cell lines express

higher levels of gal-1 after treatment with 10 μg/L of IGF-I. This new finding may point to the role of IGF-I within the extracellular milieu. Studies by other authors have shown that IGF-I leads to some changes in matrix metaloproteinase 2 levels in choriocarcinoma cells, without altering cell invasion (30). However, not much is known about ability of IGF-I to regulate gal-1 or ECM protein production.

CONCLUSION

The data presented here point to the possible role of IGF-I in cell adhesion and proliferation of choriocarcinoma cells. This study also showed that both choriocarcinoma cell lines expressed gal-1, the amount of which seems to be moderately increased by IGF-I.

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