TrkB expression level correlates with metastatic properties of L1 mouse sarcoma cells cultured in non-adhesive conditions

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Abstract

Objectives: Ability of a cell to survive without adhesion, and to overcome anoikis, is indispensable for malignant cell invasion and metastasis formation. It has previously been shown that TrkB -neurotrophin growth factor receptor might be involved in suppression of apoptosis, induced by the lack of adhesion. The aim of our study was to analyse changes in expression of genes and proteins as well as in biological properties of cancer cells cultured without adhesion. A mouse sarcoma, stable, adherent L1 cell line, derived from a spontaneously arisen Balb/c mouse lung tumour, was established *in vitro*.

Materials and methods: L1 cells resistant to anoikis were established by culture of L1 cells without adhesion, followed by selection of clones with elevated expression levels of TrkB protein. Biological characteristics of the cells were studied by migration/invasion tests and colony forming assay. Gene expression analysis was performed by with the aid of cDNA Gene Expression Array and Real-Time PCR. *In vivo* experiments were conducted in syngeneic Balb/c mice.

Results: Significant changes in gene expression, including higher expression level of TrkB, were found in cells that were able to survive without adhesion. Selected TrkB-expressing clones were found to have higher clonogenicity and invasive potential, formed more colonies in mouse lungs, and induced larger tumours, when injected subcutaneously into Balb/c mice.

Conclusion: Lack of adhesion induced significant changes in the cancer cells' behaviour, which may result from alterations in gene and protein expression levels, including changes in anoikis-connected protein – TrkB.

Introduction

Function and survival of normal cells depends on their interaction with other cells and with the extracellular matrix (ECM). Direct cell–cell and cell–matrix interactions involve activity of surface and cytoplasmic adhesion proteins and integrins, which transfer signals from the extracellular microenvironment, activate many cellular signal transduction pathways and regulate cell survival. These processes might be also regulated by signalling pathways that depend on specific growth factors.

In non-transformed cells, lack of a contact with other cells and/or ECM, induces programmed cell death, called anoikis (from Greek word meaning 'homelessness'). In physiological conditions, this process is responsible for cell homeostasis and can be induced both by extrinsic death receptors and/or by intrinsic mitochondrial apoptotic pathways. Loss of ECM contact induces translocation of BAX protein to mitochondria and this leads to cytochrome c release, followed by a caspase cascade. Activation of a cell death receptor pathway in a ligand-independent manner may also be observed. Moreover, lack of extracellular stimuli reduce activity of PI3K/Akt and MAPK signalling pathways (1,2).

Disturbances in regulation of anoikis, commonly observed in malignant tumour cells, are main cause of the cells' ability to survive in lack of adhesion and to grow in suspension. Resistance to anoikis is indispensable for tumour metastasis: intravasation to the blood or lymphatic vessels and formation of distant metastases.

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A prerequisite to cells' survival in the circulation is the ability to function in spite of lack of adhesion signals, which is called anchorage independence. This might result from deregulation of signalling pathways responsible for cell death and survival as well as from the cells' ability to aggregate.

Different molecular mechanisms have been described to be involved in acquisition of anoikis resistance by transformed cells. One of these depends on presence of somatic mutations in genes encoding proteins involved in MAPK or AKT signalling pathways. This leads to their constant activation and that results in cell survival. Overexpression of certain proteins such as TrkB – neurotrophin growth factor receptor – that activate PKB signalling, as well as of anti-apoptotic proteins such as FLIP, XIAP or PYK2 of the MAPK pathway, have also been described as prerequisites of anoikis resistance. One phenomenon of homotypic adhesion of cancer cells has been described and suggested to be involved in a mechanism of survival and proliferation, even in the absence of growth factors (3).

In this study, we have investigated a mouse L1 sarcoma cell line derived from a primary Balb/c lung tumour, and established as adhesion-dependent (4,5). These cells were characterised concerning their ability for migration, invasion, clonogenicity and potential to form subcutaneous tumours and lung metastases, in syngeneic Balb/c mice. In a previous study, we have shown that the L1 cell line had an ability to form para- and holo- clones, and we have demonstrated that the line contains cancer stem-like cells. We have also shown that a side population of these cells was more resistant to chemotherapeutic drugs than the original line, and overexpressed anti-apoptotic genes (5). Douma *et al.* (6) have proven that TrkB plays an important role in anoikis suppression. Thus, we decided to link profiles of gene and protein expression, including TrkB, in our unique L1 cell line, to its anoikis and metastatic potentials.

Materials and methods

Cells

L1 sarcoma cells derived from a primary, spontaneous Balb/c lung tumour (4,5) were cultured in DMEM supplemented with 10% foetal bovine serum and antibiotics. To study properties of cells able to survive without adhesion, the following *in vitro* models were used. First, we aimed to analyse early changes resulting from detaching cancer cells from plastic; thus, L1 cells were cultured without adhesion for 4 h on polyHema-coated dishes and cells resistant to anoikis were selected by two different experimental strategies. Cells were cultured

without adhesion for 24 h, then seeded on plastic dishes and expanded in number for the subsequent 4 days. This procedure was repeated 10 times, and the resulting population was used in further experiments. Concurrently, L1 cells were cultured without adhesion for 7 days on polyHema coated dishes, and in standard conditions on Petri dishes for the following 4 days. The procedure was repeated twice and L1 clones were derived on 24-well plates. For *in vivo* experiments, the original L1 cells served as control, and two clones with highest levels of TrkB protein were used.

The L1 cells derived from tumours and peritoneal fluids of Balb/c mice were used as *in vivo* adhesive- and non-adhesive- models, respectively. Cells were injected subcutaneously or intraperitoneally into Balb/c mice. After three to four weeks, tumours acquired sufficient volume to obtain material for further experiments. Mice were sacrificed and cells from tumours and peritoneal fluids were used for functional analysis.

Biological properties of selected L1 cells

To assess ability to form colonies *in vitro*, 10^2 L1 cells, their TrkB-positive clones, and cells grown for 24-h in suspensions were plated on Petri dishes. After 6 days, colonies were counted after fixing in methanol and staining with crystal violet.

To evaluate metastatic potential, 2×10^5 cells or their selected clones were resuspended in 0.2 ml of 0.85% saline and injected intravenously into Balb/c mice (lateral tail vein). Mice were sacrificed 21 days after tumour cell injection, lungs were excised, fixed and stained. Lung tumour colonies were counted using a dissecting microscope. To follow and measure tumour growth, an equivalent number of above described cells was injected subcutaneously into Balb/c mice.

Migration and invasion assays were carried out according to the method originally described by Repesh (7). Samples containing 2×10^4 L1 cells or their clones, in 200 μ l of serum-free medium were placed in upper compartments of BD Falcon (San Jose, CA, USA) Cell Culture Inserts or BioCoat Matrigel Invasion Chambers (BD, San Jose, CA, USA) and incubated for 40 h. Then cells from lower parts of filters were fixed in methanol and stained with Giemsa dye. Cells from each filter were counted using phase-contrast microscopy. Results were expressed as percentage of invading/migrating cells.

Molecular studies

Total RNA was extracted, using TRIzol reagent, according to the manufacturer's instructions. One microgram

of total RNA was reverse-transcribed to cDNA using Advantage RT for PCR kit. Next, gene expression (Atlas mouse 1.2 cDNA expression array, BD) was assessed and results were analysed, to identify differentially expressed genes by the Atlas Image program (Clontech, BD, Otsu, Shiga, Japan). Additionally, TrkB expression was analysed by Real-Time PCR and TrkB TaqMan Gene Expression assay (Life Technologies, Foster City, CA, USA). For western blot analysis, protein extract from the cells and their clones was used, and detection was performed using anti-TrkB (antibody recognizes full-length isoform of the protein, as well as truncated form), anti-VEGF and anti-GAPDH antibodies (Santa Cruz, CA, USA).

Results

Culturing of L1 cells for 4 h without adhesion had no significant influence on gene expression. cDNA Expression Array analysis showed only modest changes in expression of 13 genes, of which only one, immediately gene *Brf1* (butyrate response factor 1), was over-expressed in non-adherent cells. The other genes, mainly survival- and cell population growth-related (*p38*, *Plfap*, *Csk-2*, *Fgf12a*), were expressed at somewhat lower levels compared to original adherent cells (Table 1). In contrast, in cells selected after 10 detaching cycles, there were significant changes in expression of 7 genes. High expression of genes encoding cytoskeletal keratin 19 (*Ck19*) and osteopontin precursor (*Spp1*), correlated with elevated invasiveness of the cells, and high integrin beta 7 and prothymosin alpha were linked to adhesion ability

Table 1. Relative gene expression in L1 cells cultured without adhesion for 4 h, versus control (L1 cultured in standard conditions)

Gene or protein name	Without adhesion/control
Integrin-linked kinase (ILK); integrin-binding protein kinase	0.02
Ezrin; villin 2	0.02
47-kDa heat shock protein precursor (HSP47)	0.02
Glutathione S-transferase 5 (GST5-5); GST mu (GSTM2)	0.03
Fibroblast growth factor 12-related protein (FGF12A)	0.03
Mitogen-activated protein kinase p38 (MAP kinase p38)	0.03
45-kDa calcium-binding protein precursor (CAB45)	0.03
Semaphorin G Precursor (SEMAPHORIN G)	0.03
S100 calcium-binding protein A1	0.03
Osteopontin precursor (OP); bone sialoprotein 1	0.28
Proliferation-associated protein 1 (PLFAP)	0.35
Cyclin-dependent kinases regulatory subunit 2 (CKS-2)	0.45
Butyrate response factor 1	60.0

Atlas mouse 1.2 cDNA expression array has been used to obtain the data presented in Tables 1–3.

and proliferation potential. Significant repression of transcription was found for paired related homeobox 2 (*Pmx2*) gene (Table 2). As the Atlas mouse 1.2 cDNA expression array does not contain the probe for *TrkB*, this gene was included in the Real-Time PCR experiments (6). *TrkB* expression was found to be 8.2 times higher in sequentially detached cells compared to controls (Fig. 1). Moreover, repeated detachment of cells and growth in suspension influenced their colony formation ability, as shown by plating efficiency results. Detached cells had higher clonogenicity (66.9 ± 9.4 and 79.6 ± 18.7 colonies after 5 and 10 rounds of detachment, respectively) than control original L1 cells

Table 2. Relative gene expression in L1 cells cultured periodically (10×) without adhesion for 24 h, versus L1 control cells

Gene or protein name	Without adhesion/control
Paired mesoderm homeobox protein 2 (PMX2; PRX2); S8	0.21
Prothymosin alpha (PTMA)	6.44
Integrin beta 7	12.22
Insulin-like growth factor binding protein -6 (IGFBP 6)	15.40
Osteopontin precursor (OP); bone sialoprotein 1; minopontin; early T-lymphocyte activation 1 protein (ETA1); secreted phosphoprotein 1 (SPP1); calcium oxalate crystal growth inhibitor protein	15.77
Type I cytoskeletal keratin 19 (CK19; KRT19; K19); cytokeratin 19	20.03
Transcription termination factor 1 (TTF1)	62.55

Figure 1. The results of Real-Time PCR analysis of TrkB expression level performed on L1 control cells, selected L1 clones, cells isolated from peritoneal fluid and from solid tumour grown in Balb/c mice. Relative mRNA levels (probe/control) are presented in logarithmic scale. Asterisk (*) represents statistical significance of 10× detached cells, clone 4, clone 6, cells from peritoneal fluid and cells from solid tumour versus control L1 cells.

cultured in standard conditions (50.9 ± 8.95). Observed differences were found to be statistically significant ($P < 0.01$).

In addition, to verify changes induced by lack of adhesion, the *in vivo* model for adhesive and non-adhesive cells including L1 cells derived from solid subcutaneous tumours and peritoneal fluids was examined. Gene expression analysis revealed significant differences for 9 genes and 6 genes respectively, from peritoneal fluid cells and solid tumour cells, (Table 3). Moreover, as compared to original L1 cells, *TrkB* mRNA level increased 10-fold in peritoneal fluid cells and reached massive, 750 times higher values in solid tumour cells (Fig. 1).

Clones established after two 7-day cultures of L1 cells on polyHema-coated dishes presented up to 4-fold higher *TrkB* expression (Fig. 1), which contributed to the elevated protein level as shown by western blot analysis (Fig. 2). Two of the clones (4 and 6) were chosen for the further *in vitro* and *in vivo* studies. First, clonogenicity and invasiveness of TrkB-expressing L1 clones were examined, and revealed their higher invasive potential compared to original cells (Fig. 3a and 3b).

In vivo studies revealed that clones with high TrkB expression had higher potential for tumour formation

Table 3. Relative gene expression in L1 cells derived from peritoneal fluid (p.f.) versus solid tumour cells (s.t.)

Gene or protein name	p.f./s.t.
Maspin precursor; protease inhibitor 5 (PI5)	0.022
Insulin-like growth factor binding protein-6 (IGFBP 6)	0.067
Type I cytoskeletal keratin 18 (KRT1-18; KRT18)	0.067
Transcription termination factor 1 (TTF1)	0.25
Ezrin; villin 2	0.26
Transcriptional co-activator of AML-1 & LEF-1 (ALY)	0.27
Heat shock 86-kDa protein	2.1
Recombination activating protein 1 (RAG1); RGA	2.43
Glucose-6-phosphate isomerase (GPI); neuroleukin (NLK)	2.61
Basigin precursor, extracellular matrix metalloproteinase inducer	2.81
Tyrosine-protein kinase ryk precursor; kinase vik; nyk-R	4.21
Stra14 – basic-helix-loop-helix protein.	5.24
Cathepsin L precursor (CTSL); major excreted protein (MEP)	6.04
Growth arrest & DNA-damage-inducible protein 153 (GADD153)	15.85
Paired mesoderm homeobox protein 2 (PMX2; PRX2)	Up ^a

^aPresent in peritoneal fluid cells, absent in solid tumour cells.

than original L1 cells. After subcutaneous cancer cell injection, of either L1 clone 4 or L1 clone 6, all mice

Figure 2. The results of Western blot analyses of TrkB, its truncated form (TrkB.T1 95 kDa) and VEGF performed on L1 control cells, selected L1 clones (clone 4, clone 6) and L1 cells isolated from peritoneal fluid (p.f.) and from solid tumour (s.t.) grown in Balb/c mice. The GAPDH protein was used as a control reference.

Figure 3. The biological properties of L1 cells: invasiveness (a) plating efficiency (b) and mice tumour volume (c) of L1 control cells and selected L1 clones. (a) mean % cells passed matrigel/cells passed membrane in Transwell $\pm$ SD, $P \leq 0.01$. (b) mean number of colonies per dish $\pm$ SD, $P \leq 0.01$. (c) mean volume $\pm$ SD, the differences between injected clones and control are statistically significant, $P \leq 0.01$. Asterisk (*) represent statistical significance of L1 clone 4 versus L1 control; (**) represents statistical significance of L1 clone 6 versus L1 control. The results are statistically significant, $P \leq 0.01$.

were diagnosed with tumours on day 13. A single mouse from the L1 control group developed a tumour on day 16, but all remaining mice had tumours by day 20. Moreover, tumours that had arisen from TrkB-expressing clones grew faster, and because of their tumour volume, recipient mice had to be sacrificed after 22–27 days; subcutaneous injection of cells induced smaller tumours with lower growth rates (Fig. 3c) and created less colonies in the lungs after intravenous injection, than TrkB-expressing clones. Lung colony assay showed that after injection of cells of TrkB-expressing clone 6, there were 66.5 ± 27.14 metastases compared to 16.5 ± 8.18 following injection of L1 cells, ($P < 0.05$). Of all mice injected with the TrkB-expressing clone, 4 died before the end of the experiment (3 weeks after injection).

Discussion

Anoikis resistance is an important step during cancer progression. The aim of this study was to determine whether selection or induction of cells to grow in suspension might contribute to higher invasive and metastatic potential. First, we showed that tumour L1 cell clones, selected by induced non-adhesive cell population growth, as well as by repeated detachment, had higher expression of *TrkB* mRNA and protein, compared to L1 cells grown in adhesive conditions. Increased TrkB levels were also observed in cells selected from both

peritoneal fluids and tumour masses of Balb/c mice. This was consistent with higher invasiveness and clonogenicity of cells observed *in vitro*. Moreover, injections of mice with TrkB-expressing L1 clones resulted in higher numbers of lung colonies and in rapidly proliferating subcutaneous tumours that led to earlier death of host animals. Compared to control L1 cells, established clones, as well as peritoneal fluid and tumour cells presented also higher levels of VEGF protein. These findings are consistent with studies by Au *et al.* performed on ovarian carcinoma and ovarian cancer cell lines, and support the theory that activation of TrkB induces overexpression of VEGF. Thus, there is possible interconnection between these two pathways (8,9).

TrkB protein, a member of the tropomyosin-related kinase (Trk) family of neurotrophin receptors, was first described by Douma *et al.* (6) as protein with a special role in anoikis resistance. In functional genomic screening of rat intestinal epithelial (RIE) cells transfected with retroviral complementary DNA library, in search of potential suppressors of anoikis, TrkB expression was associated with cells' resistance to apoptosis induced by lack of adhesion. RIE cells, stably transfected with TrkB expressing vector, displayed disturbances in organisation and loss of cell–cell contact in a confluent state. Moreover, after reaching confluence, cells continued to proliferate, formed spheroids and grew in suspension. Further experiments confirmed that TrkB, together with its ligand BDNF (brain-derived neurotrophic factor),

suppressed anoikis in detached cells by direct activation of phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathway (10). Silencing TrkB expression by siRNA or by inhibition of the PI3K/PKB signalling pathway, with LY294002, reduced cancer cell invasion and migration and enhanced apoptosis, as shown in various other cell lines for example, ovarian cancer cell lines (6,8,11).

TrkB over-expression is observed in many malignancies, including neuroblastomas, pancreatic, ovarian and prostate carcinomas, lung and colon cancers and other tumours of epithelial or lymphoid origin. In some cases, TrkB over-expression correlates with higher metastatic potential, confirmed by presence of distant metastases (10,12–14). Detailed mechanisms have not yet been explained, but some evidence suggests that TrkB is an important factor in this process (9,15,16). In neuroblastoma and ovarian cancer, activation of TrkB by BDNF has been shown to induce VEGF expression. TrkB receptor stimulation, dimerization and autophosphorylation leads not only to triggering of PI3K/PKB pathway but also to induction of Ca^{2+} release and activation of PLC γ circuits, or enhanced signalling via RAS/MAPK. However, only PI3K/PKB activation results in inhibition of pro-apoptotic signalling. For example, activated AKT phosphorylates BAD protein (an inhibitor of anti-apoptotic Bcl-X_L) leading to its inactivation or FKHRL1 – a transcription factor involved in expression of *Bim* and *FASL* genes encoding pro-apoptotic proteins (17).

One process related to anoikis resistance is epithelial-mesenchymal transition (EMT). Epithelial-mesenchymal transition not only leads to significant changes in cell phenotype but also induces ECM reorganization that promotes cell proliferation and metastatic growth. During EMT, increased expression of ECM elements, such as fibronectin and vimentin, along with an increase in levels of growth factors and matrix metalloproteinases is observed. Together with neoangiogenesis, these processes are important for further tumour growth and progression. EMT involves transcription factors, for example SNAIL or SIP1, main downregulators of *E-cadherin* gene. Moreover, formation of KAP-1/ CBF-A /FSP1 complex increases expression of several EMT markers such as N-cadherin, SNAIL and TWIST proteins. EMT leads to higher motility of cancer cells which promotes invasion and metastasis formation (18,19).

Recently, TrkB has also been described as a positive regulator of EMT, especially with respect to expression of two crucial regulators of this process – SNAIL and TWIST – activated by phosphorylated AKT. Studies performed on cell lines, derived from head and neck squamous cell carcinoma, revealed that TrkB over-

expression results in upregulation of mesenchymal molecular markers (20).

Here, we have shown that tumour cells, following non-adherent growth have different gene expression profile compared to the relevant control cells cultured under standard conditions. Expression of genes related to proliferation, differentiation, apoptosis and metastatic potential were identified as significantly changed. Among them, we found elevated cytoskeletal keratin 19 (*Ck19*) and osteopontin precursor, both linked to increased cell invasion potential and integrin beta 7 related to adhesion, and proliferation regulator – prothymosin alpha.

Gene expression profiling revealed differences between an *in vivo* models of adherent and non-adherent L1 cells. Compared to cells derived from solid tumours, cells derived from peritoneal ascities, presented elevated mRNA levels of genes related to proliferation, apoptosis inhibition (*HSP* and *GPI*) and metastasis (*emmprin* and *STRA14* transcription factor). In humans, *Strat14* (human orthologue *DEC1*) is generally considered to be a suppressor of anchorage-independent growth (21); however, it has also been shown to play a critical role in TGF- β -mediated survival of breast carcinoma cells, as well as in cancer progression (22). Additionally, cells of solid subcutaneous tumours over-expressed genes encoding villin, which is responsible for cell adhesion, maspin precursor and cytokeratin 18, which correlates with reduced tumour malignancy.

To generalise roles of specific genes/proteins in adherent and non-adherent cell growth in the context of tumorigenic potential of the cells, further studies (including other cell lines), are necessary. Our results provide grounds for studies on TrkB involvement in suppression of apoptosis (anoikis) and metastatic spread, including shRNA experiments, and attempts at gene therapy *in vivo*. To achieve such an objective, it is essential to carry out studies on a relevant model of adherent and non-adherent cancer cell growth *in vivo*, such as the syngeneic, transplantable model of L1 cells derived from the Balb/c mouse, and propagated *in vivo* the model described in previous and present studies.

To summarise, lack of adhesion induces significant changes in L1 mouse lung sarcoma cells. Changes in biology of these cells may result from discovered alterations in gene and protein expression levels, including changes in anoikis-related protein TrkB.

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