Vitronectin, fibronectin and epidermal growth factor induce proliferation via the JNK and ERK pathways in insulinoma INS-1 cells

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Abstract An insulinoma is a tumor formed by beta cells in the Langerhans islets of the pancreas. Vitronectin (VTN), fibronectin (FN) and epidermal growth factor (EGF) are important in cell signaling. The aim of this study was to investigate the molecular mechanism that occurs in INS-1 cells with the administration of VTN, FN and EGF in proliferative doses. We determined the proliferative doses of EGF, VTN and FN. The molecular mechanism of proliferation has been investigated alone or in the combination of these proteins. It was observed that INS-1 cells did not have VTN and FN. Cell viability increased with the administration of 0.1 µg/ml VTN, 0.1 µg/ml FN and 1 mg/ml EGF. Proliferation increased with the administration of FN + EGF, and VTN + FN + EGF together when compared to the control group. The total JNK levels did not change between the groups; however, the active JNK levels increased in the VT + FN + EGF group compared to the control group. The total ERK levels increased in the VT + FN + EGF group, and the active ERK levels

increased in the VTN + FN, VTN + EGF and VTN + FN + EGF groups compared to the control group. The JNK and ERK pathways are important for proliferation. The JNK and ERK pathways were activated in VTN + FN + EGF administered group. However, it was observed that the ERK pathway was more active than the JNK pathway.

Keywords Insulinoma · INS-1 cells · Vitronectin · Fibronectin · Epidermal growth factor · JNK/ERK pathways

Introduction

It is not easy to use primary beta cells in biochemical and molecular studies because it is difficult to obtain pancreatic endocrine tissue. Therefore, insulin-releasing beta-cell lines are used instead of primary beta cells. These cell lines are commonly RIN, HIT, beta TC, MIN6 and INS-1 cells, and they are highly important for investigating the molecular mechanism of beta cells (Poitout et al. 1996). INS-1 cells among these cell types have important characteristics of pancreatic beta cells (Skelin et al. 2010).

Integrins are a family of cell–cell adhesion molecules and extracellular matrix proteins. Laminin, collagen, fibronectin (FN), and vitronectin (VTN) are extracellular matrix proteins. Furthermore, integrins

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include cell surface receptors (Hynes 1987). The collagen-binding or heparin-binding domains of VTN bind to the extracellular matrix. VTN interacts with integrins. In this way, cell adhesion, spreading, and migration are promoted (Hess et al. 1995; Huang et al. 1998). Some researchers think that VTN is associated with growth factor signals. This interaction may create a mechanism between growth factors and extracellular matrix proteins. This mechanism is important for promoting cell growth (Schvartz et al. 1999).

Integrins are required not only for their own signal but also for the ideal activation of growth factor receptors such as Epidermal Growth Factor (EGF) (Giancotti and Ruoslahti 1999; Wang et al. 2008). There are studies showing that integrin in many cell lines is primarily associated with specific growth factor receptors (Wang et al. 2008).

Only a limited amount of information is available on the actions of EGF and integrins on the proliferation of insulinoma cells. The effect of EGF on the proliferation of insulinoma cells was studied with the cell lines RINm5F and 5AH (Nielsen 1989; Sjöholm 1995). Only a small effect was observed. The INS-1 cell line has yet to be studied. As proteins in the extracellular matrix, VTN and FN were evaluated for their role in the proliferation of FG pancreatic cancer cells, with only a weak effect being observed. No study of exogenously added VTN and FN on insulinoma cell proliferation has been described.

MAPKs regulate extracellular signals and consist of three well-characterized subfamilies in multicellular organisms. These are ERK1 and ERK2; c-Jun NH₂-terminal kinases, JNK1, JNK2, and JNK3; P38 enzyme, p38 α , p38 β , p38 γ , and p38 δ (Johnson and Lapadat 2002). JNK stimulates cell proliferation or apoptosis in different cell types and activates these stimuli (Janjic and Wollheim 1992). ERK 1/2 pathway plays an important role in cell proliferation, signal transduction stimulating growth and differentiation. The pathology, progression and oncogenic behavior of human cancers such as non-small cell lung cancer, breast cancer, head and neck squamous cell carcinoma and colorectal cancer are related to the activation of the ERK pathway (de Bono and Rowinsky 2002; Santen et al. 2002; Adjei 2005; De Luca et al. 2012).

The aim of this study was to investigate the role of VTN, FN, and EGF in insulinoma INS-1 cells and the

molecular mechanisms behind their actions on proliferation.

Materials and methods

Cell line

The rat insulinoma cell line INS-1 was a generous gift from Prof. Dr. Claes B. Wollheim (University Medical Center, Geneva). INS-1 cells were cultured in a humidified atmosphere containing 5% CO₂ in RPMI 1640 (Invitrogen) containing 10 mM HEPES (Sigma), 50 μ M 2-mercaptoethanol (Biorad), 1 mM sodium pyruvate (Sigma), 5% fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Gibco). INS-1 cells were passaged once a week by gentle trypsinization with 0.25% trypsin–EDTA (Sigma).

Determination of medium for given substances to insulinoma INS-1 cells

INS-1 cells were incubated in either the RPMI medium containing the fetal calf serum (FCS), RPMI medium without FCS, 1% Bovine Serum Albumin (BSA) or phosphate buffered saline (PBS) for the purpose of investigating the presence or absence of FN, VTN and EGF for 24 h at 37 °C in a 5% CO₂ incubator. We determined cell viability colorimetrically by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Western blotting

10⁶ cells/wells were plated on 6-well plates and incubated in an incubator with 5% CO₂ at 37 °C until the cells proliferated. VTN and FN were applied to the cells for 24 h. The cells were removed by 0.25% trypsin/EDTA and lysed. A phosphatase-proteinase inhibitor cocktail was added to the samples. 40 μ g protein loaded to 7.5% gel SDS-PAGE and proteins were separated. VTN and FN were loaded as positive control. Afterward, proteins were transferred to the PVDF membrane using a wet transfer method. The membranes were incubated at room temperature for 1 h with the block (%5 skim milk in TBST), following which the primary antibody (for VTN-sc-74485 and for FN-sc-8422) was applied for overnight at 4 °C.

Afterward, membranes were incubated with the secondary antibody (anti-mouse sc-358914) for 1 h at room temperature. The membrane was treated with luminol reagent (sc-2048) for 5 min at room temperature, and the bands were made visible with a Gel Logic 1500 Imaging System Device. Data analysis was performed with Kodak Carestream MI analysis program. Beta-actin was used to normalize cytoplasmic proteins, while Ponceau S was used for the normalization of secretion protein.

Cell viability assay

We determined the effects of VNT (0.05–10 $\mu\text{g/ml}$), FN (0.05–10 $\mu\text{g/ml}$) and EGF (5–2500 ng EGF) colorimetrically by using MTT. The cells were seeded at a density of 5×10^4 cells/well in 96 well plates and incubated in 5% CO_2 and 95% humidified air at 37 °C. On the next day, VTN, FN or EGF were administered exogenously to cells and the cells were incubated in 5% CO_2 /95% humidified air at 37 °C for 24 h. After incubation, the MTT (0.5 mg/ml) (Applichem, Darmstadt, Germany) assay was evaluated in 5% CO_2 /95% humidified air at 37 °C for 2 h and following the removal of the medium, isopropanol was added to solve formazan (Janjic and Wollheim 1992). The optical density was recorded by a spectrophotometer at 540 nm. The cell viability rate was calculated as % of control.

Cell proliferation assay

We determined cell proliferation using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche, Basel, Switzerland). The control values were accepted as 100%, and cell proliferation values were calculated.

JNK, p-JNK, ERK and p-ERK activity assay

The relative amount of JNK and p-JNK was measured using the RayBio® Cell-Based JNK (Thr183/Tyr185) ELISA Kit, while the relative amount of ERK and p-ERK was measured using the RayBio® Cell-Based ERK1/2 (Thr202/Tyr204) ELISA Kit (RayBiotech, Inc., Norcross, GA, USA). The OD values were obtained, and the JNK, p-JNK, ERK and p-ERK values were calculated.

Statistical analysis

Statistical analyses were performed with minimum triple data by the ANOVA variance analysis test and nonparametric Tukey's test using GraphPad Prism 5 computer program. Results were reported as mean $\pm$ SEM. *P* values less than 0.05 were considered to be significant.

Results

Insulinoma INS-1 cells incubated with RPMI medium not containing the fetal calf serum

The medium containing FCS was compared to the medium without FCS. Cells in the RPMI medium without FCS showed near-viability to cells incubated in the RPMI medium containing 5% FCS (Fig. 1). Therefore, VTN and FN were administered to cells in the RPMI medium without FCS and incubated for 24 h.

There were not VTN and FN in insulinoma INS-1 cells incubated at RPMI medium not containing FCS

There is no study on the exogenous administration of VTN and FN in insulinoma cells. The effect of these proteins in insulinoma INS-1 cells is unknown. We found out that there is no FN in the cell lysate according to Western blotting results. VTN is present in cell lysates incubated in the medium containing FCS, while it is not present in cell lysates incubated in media without FCS. The found VTN originates from the FCS in the medium. As a result, VTN and FN are not found in the cell (Fig. 2a).

5% FCS containing RPMI medium and incubated cell secretion with 5% FCS containing RPMI medium contains VTN. The RPMI medium also contains VTN. Therefore, the source of VTN observed in cell secretion is the RPMI medium. There is VTN in the secretions of the cells incubated in the RPMI medium lacking FCS because of the RPMI medium. As a result, the RPMI medium lacking FCS and the RPMI medium containing it have the same level of VTN. Therefore, we can say that there is no VTN in insulinoma INS-1 cell secretion (Fig. 2b).

Fig. 1 Image and cell viability % of insulinoma INS-1 cells in RPMI medium containing 5% FCS (I); 100 ± 0 ; RPMI medium not containing FCS (II); 92.71294 ± 21.88 ; RPMI medium containing 1% BSA (III); 61.25036 ± 9.5 and PBS (IV); 1.872463 ± 0

Fig. 2 **a** Amount of Vitronectin (VTN) and Fibronectin (FN) in insulinoma cell lysate. L1, L2, L3: insulinoma cell lysate incubated in RPMI medium containing 5% FCS, L4, L5: insulinoma cell lysate incubated RPMI medium not containing

FCS. **b** Amount of VTN and FN in insulinoma cell secretion. S1, S2, S3: insulinoma cell secretion incubated in RPMI medium containing 5% FCS, S4, S5, S6: insulinoma cell secretion incubated in RPMI medium not containing FCS

Determination of cell viability with exogenously given of FN, VTN and EGF on insulinoma INS-1 cells

Cell viability was shown colorimetrically by using MTT. The graphs are shown for FN (Fig. 3a), VTN (Fig. 3b) and EGF (Fig. 3c). Cell viability was observed at the highest level with the exogenous administration of 0.1 μg FN, 0.1 μg VTN and 1000 ng EGF. Therefore, it was decided to give 0.1 μg FN, 0.1 μg VTN and 1000 ng EGF to INS-1 cells.

Proliferation increased when given combine VTN, FN and EGF in insulinoma INS-1 cells

Cell proliferation was measured with spectrophotometry. The graph obtained is presented (Fig. 4). When the groups were compared between each other in term of cell proliferation, a statistically significant result could be obtained ($P_{\text{ANOVA}} < 0.05$). As a result of binary comparisons, it was determined that cell proliferation increased significantly in the group that was administered with FN + EGF ($P < 0.05$) and

Fig. 4 Cell proliferation % of insulinoma INS-1 cells. Control 100 ± 0.01667 ; Vitronectin (VTN) 104.2 ± 10.32 ; Fibronectin (FN) 106.8 ± 6.091 ; epidermal growth factor (EGF) 105.5 ± 5.786 ; VTN + FN 102.4 ± 8.102 ; VTN + EGF 106.8 ± 5.533 ; FN + EGF 108.4 ± 3.253 ; VTN + FN + EGF 116.4 ± 4.714 . ^a $P < 0.05$ versus control group, ^b $P < 0.01$ versus control group

VTN + FN + EGF ($P < 0.01$) when compared to the control group.

Fig. 3 **a** Cell viability % of insulinoma INS-1 cells in different doses of Fibronectin (FN). Control 100 ± 0 ; 0.05 μg FN 107.9381 ± 5.322 ; 0.1 μg FN 110.667 ± 7.172 ; 1 μg FN 90.69269 ± 6.11 ; 10 μg FN 104.1431 ± 7.447 . **b** Cell viability % of insulinoma INS-1 cells in different doses of Vitronectin (VTN). Control 100 ± 0 ; 0.05 μg VTN 103.5958 ± 3.163 ; 0.1 μg VTN 106.1347 ± 2.693 ; 1 μg VTN 105.6285 ± 2.06 ; 10 μg VTN 92.19857 ± 3.687 . **c** Cell viability % of insulinoma INS-1 cells in different doses of

epidermal growth factor (EGF). Control 100 ± 0 ; 5 ng EGF 105.3474 ± 10.9 ; 10 ng EGF 110.8284 ± 4.34 ; 15 ng EGF 107.9381 ± 5.322 ; 25 ng EGF 105.2864 ± 4.825 ; 50 ng EGF 108.7687 ± 6.578 ; 100 ng EGF 108.9212 ± 1.957 ; 150 ng EGF 104.109 ± 4.858 ; 250 ng EGF 107.2011 ± 3.01 ; 500 ng EGF 105.8385 ± 3.401 ; 1000 ng EGF 118.6662 ± 6.144 ; 1500 ng EGF 99.05728 ± 1.831 ; 2000 ng EGF 98.07118 ± 10.2 ; 2500 ng EGF 100.3618 ± 0.4706

ERK pathway is more active from than JNK pathway when given combine VTN, FN and EGF in insulinoma INS-1 cells

The relative amount of JNK, p-JNK, ERK and p-ERK was measured using the ELISA Kit with spectrophotometry. The graph is presented for JNK, p-JNK (Fig. 5a) and ERK, p-ERK (Fig. 5b).

Considering JNK, when the groups were compared between each other, a statistically significant result could not be obtained ($P_{ANOVA} > 0.05$). There was no statistically significant change in JNK amounts as a result of binary comparisons ($P > 0.05$).

Considering p-JNK, when the groups were compared between each other, a statistically significant result could not be obtained ($P_{ANOVA} > 0.05$). As a result of binary comparisons, the p-JNK amount increased significantly in the group that was administered with VTN + FN + EGF when compared to

Fig. 5 a JNK and p-JNK levels of insulinoma INS-1 cells. For JNK, control 0.3658 ± 0.02324 ; vitronectin (VTN) 0.3690 ± 0.009065 ; fibronectin (FN) 0.3850 ± 0.008396 ; epidermal growth factor (EGF) 0.3855 ± 0.01390 ; VTN + FN 0.3953 ± 0.01537 ; VTN + EGF 0.3845 ± 0.03118 ; FN + EGF 0.4090 ± 0.0130 ; VTN + FN + EGF 0.4530 ± 0.04424 ; for p-JNK, control 0.1880 ± 0.02351 ; VTN 0.1850 ± 0.01409 ; FN 0.1873 ± 0.004589 ; EGF 0.1943 ± 0.01440 ; VTN + FN 0.2130 ± 0.02159 ; VTN + EGF 0.2073 ± 0.009068 ; FN + EGF 0.2280 ± 0.02214 ; VTN + FN + EGF 0.2580 ± 0.02127 . For ERK, control 0.4573 ± 0.05325 ; VTN 0.4323 ± 0.02548 ; FN 0.4120 ± 0.02719 ; EGF 0.4658 ± 0.01657 ; VTN + FN 0.4675 ± 0.01320 ; VTN + EGF 0.4833 ± 0.01485 ; FN + EGF 0.4665 ± 0.006384 ; VTN + FN + EGF 0.5740 ± 0.04681 ; for p-ERK, control 0.1095 ± 0.009629 ; VTN 0.09733 ± 0.004828 ; FN 0.1075 ± 0.005175 ; EGF 0.1100 ± 0.007132 ; VTN + FN 0.1148 ± 0.005406 ; VTN + EGF 0.1158 ± 0.003655 ; FN + EGF 0.1085 ± 0.005233 ; VTN + FN + EGF 0.1378 ± 0.01104 . For ERK, ^a $P < 0.05$ versus VTN group, ^b $P < 0.05$ versus FN group, ^c $P < 0.01$ versus EGF group, ^d $P < 0.05$ versus VTN + FN group, ^e $P < 0.05$ versus FN + EGF group. For p-ERK, ^a $P < 0.05$ versus control group, ^b $P < 0.05$ versus VTN group, ^c $P < 0.01$ versus VTN group, ^d $P < 0.05$ versus FN group, ^e $P < 0.05$ versus EGF group, ^f $P < 0.05$ versus VTN + FN group, ^g $P < 0.05$ versus VTN + EGF group, ^h $P < 0.05$ versus FN + EGF group

the control group ($P < 0.05$), VTN group ($P < 0.05$), FN group ($P < 0.01$), EGF group ($P < 0.05$) and VTN + EGF group ($P < 0.05$).

Considering ERK, when the groups were compared between each other, a statistically significant result could not be obtained ($P_{ANOVA} > 0.05$). As a result of binary comparisons, the ERK amount increased significantly in the group that was administered with VTN + FN + EGF when compared to the VTN group ($P < 0.05$), FN group ($P < 0.05$), EGF group ($P < 0.01$), VTN + FN group ($P < 0.05$) and FN + EGF group ($P < 0.05$). The ERK amount increased significantly in the FN + EGF group when compared to the FN group ($P < 0.05$).

Considering p-ERK, when the groups were compared between each other, a statistically significant result could not be obtained ($P_{ANOVA} > 0.05$). As a result of binary comparisons, the p-ERK amount increased significantly in the VTN + FN + EGF group when compared to the control group

($P < 0.05$), VTN group ($P < 0.01$), FN group ($P < 0.05$), EGF group ($P < 0.05$), VTN + FN group ($P < 0.05$), VTN + EGF group ($P < 0.05$) and FN + EGF group ($P < 0.05$). The p-ERK amount increased significantly in the VTN + FN group ($P < 0.05$) and VTN + EGF group ($P < 0.05$) when compared to the VTN group.

Discussion

Insulinoma INS-1 cells mimic the function of normal pancreatic beta cells with some features such as high insulin content and responsiveness to glucose. Therefore, insulinoma INS-1 cells are preferred for studies associated with pancreatic beta cells (Skelin et al. 2010). An insulinoma is a pancreatic tumor derived from pancreatic beta cells. The invasion of adjacent structures occurs in pancreatic cancer. Tumor cell invasion is characterized by the loss of junctional contact in the tumor tissue. This situation occurs via the disrupted relationship of the cell–cell and cell–extracellular matrix (Ellenrieder et al. 1999). VTN and FN are extracellular matrix proteins. VTN is a glycoprotein that is produced primarily in the liver (Seiffert et al. 1994). It is secreted into the blood and found in the serum and extracellular matrix. Circulating VTN is found as a monomer, while cell-bound VTN is found as multimeric (Zhuang et al. 1997; Wilkins-Port and McKeown-Longo 1998; Singh et al. 2010). VTN plays an important role in wound healing and clotting (Podor et al. 2002). It also plays a role in many biological processes such as cell adhesion and migration (Singh et al. 2010). FN is an important protein with the adhesive property in the body. Fibrils are made from fibronectin. It attaches cells to the extracellular matrix that surrounds them. Fibronectin fibrils also control cell behavior. There is no study on VTN and FN in insulinoma cells. The effect of these proteins in insulinoma INS-1 cells is unknown. Mammalian development and fertility have not exhibited any negative effect in case of the complete absence of VTN. These data suggest that it is tolerated during the provision of critical developmental functions or is compensated by other extracellular matrix proteins (Zheng et al. 1995). Contrary to this, the disruption of the FN gene causes a major defect in embryogenesis (George et al. 1993). EGF and EGFR are important for cell proliferation in the pancreas.

EGFR is particularly important in the embryonic period and pancreatic development. This situation has been shown in EGFR knockout and transgenic mouse models. EGFR is fatal for the embryonic period when it is genetically impaired (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995; Miettinen et al. 2000; Wang et al. 2010). In the studies conducted, EGF was exogenously administered to cells (Kawahara et al. 2002) while VTN and FN were used as adhesive ligands (Wu et al. 2011; Correia et al. 2017).

The first major goal when working with cancer cells is to kill cancer cells. It is aimed at detecting substances that kill cancer cells and treating them accordingly. Another approach to cancer cell research is to find substances that cause proliferation, and prevent cell proliferation by blocking these substances. As is known, EGF is an important growth factor that plays a role in proliferation. Vitronectin and fibronectin are used as cell adhesion proteins. When they are used as cell adhesion proteins, they cause cell adhesion and this stimulates proliferation. In other studies, these substances are added to plates in advance to provide the adhesion of cells. However, it is not known how it will work as a result when added to the cell. We thought what effect would be shown in case of the exogenous administration of EGF, VTN and FN to cells. There is no study on the exogenous administration of these proteins to cells in insulinoma INS-1 cells. The effects of VTN, FN, and EGF on cell viability were investigated for the first time in insulinoma INS-1 cells. Proliferative doses were found in case of the exogenous administration of VTN, FN, and EGF, separately. Furthermore, cell proliferation in INS-1 cells was observed when the combination of VTN, FN, and EGF was administered exogenously.

Integrins and growth factor receptors cooperate by different mechanisms. There are pathways regulated by integrins and growth factor receptors. These pathways are the PI3K/Akt, MEK/ERK, and small GTPase (Rho and Rac) pathways (Grassian et al. 2011; Moreno-Layseca and Streuli 2014). MAP kinases containing ERK (Schaeffer and Weber 1999), p38 MAP kinase (Han and Ulevitch 1999) and JNK (Robinson and Cobb 1997; Davis 2000; Chang and Karin 2001) regulate cell activity in many events such as gene expression, mitosis, and apoptosis in the cell (Roy et al. 2010). ERK activates growth factors such as EGF (Lubinus et al. 1994), while JNK induces

events such as gene expression and apoptosis (Davis 2000; Ono and Han 2000; Chang and Karin 2001). In our previous study, it was observed that cell death was mediated by the JNK pathway with the exogenous administration of 4-MC in INS-1 cells (Karatug Kacar and Bolkent 2018). Except for our recent study, the JNK/ERK pathway was not investigated by the exogenous administration of VNT, FN, and EGF in insulinoma INS-1 cells.

As is known VTN and FN bind to their specific integrins, and EGF binds to EGFR. The previous studies have shown that integrins are activated by EGFR (Wang et al. 2008). However, the effect of activated EGFR on integrins has not been clearly explained. When integrins and growth factor receptors are active, cells proliferate. Therefore, the presence of these proteins will activate their receptors and the activation of the receptors will trigger cell proliferation. However, it is not known what an effect these proteins will have alone or in their combination in this interaction.

In the present study, no change was observed in the JNK level when VTN, FN and EGF were administered separately to cells, whereas an increase in the p-JNK level was observed when VTN, FN, and EGF were administered to cells. Similarly, the ERK levels increased in FN + EGF and triple combination while the ERK levels remained unchanged when VTN, FN, or EGF were administered to cells separately. At the same time, the p-ERK levels also increased when VTN + FN, VTN + EGF were administered to cells, as well as in case of a triple combination. Phosphorylation of the JNK and ERK pathways is important in insulinoma INS-1 cells. We studied both the total form and phosphoryl form because we wanted to see the total JNK and ERK levels, and phosphorylated of how much level of total JNK and ERK. Both active JNK and ERK are important in insulinoma INS-1 cells in proliferation. However, ERK is more active than JNK for a given triple combination, and shows more significant results than JNK.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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