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Essential role of Notch signaling in apoptosis of human pancreatic tumoral cells mediated by exosomal nanoparticles

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We previously reported that exosomal nanoparticles secreted by human pancreatic tumoral cell lines decrease tumoral cell proliferation through the mitochondria-dependent apoptotic pathway, because of activation of pro-apoptotic phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and of glucose synthase kinase-3β (GSK-3β). Interactions between exosomal nanoparticles and cells are thought to involve membrane lipid rafts. However, the underlying mechanism is unknown. Here, we report that the interaction of exosomal nanoparticles with pancreatic cancer cells led to decreased expression of hairy and enhancer-of-split homolog-1 (Hes-1), the intranuclear target of Notch-1 signaling pathway, and to activation of the apoptotic pathway after a cell cycle arrest in G_0G_1 phase. Strikingly, the expression level of Notch-1 pathway components was critical, because exosomal nanoparticles decreased the proliferation of cells in which these partners are either weakly represented, in differentiated adenocarcinoma cells, or inhibited, in poorly differentiated carcinoma cells, by blocking presenilin in the γ-secretase complex that regulates the Notch-1 pathway. Overexpression of Notch-1 intracellular domain resulted in the reversion of the cell proliferation inhibition promoted by exosomal nanoparticles. Blocking presenilin unexpectedly resulted in activation of PTEN and GSK-3β. Conversely, inhibiting either PTEN or GSK-3β increased Hes-1 expression and partially counteracted the inhibition of proliferation promoted by exosomal nanoparticles, highlighting reciprocal regulations between Notch signaling and PTEN/GSK-3β. We concluded that interactions of exosomal nanoparticles with target cells, at lipid rafts where Notch-1 pathway partners are localized, hampered the functioning of the Notch-1 survival pathway and activated the apoptotic pathway, which determines tumoral cell fate.

Key words: apoptosis; exosomes; Notch signaling; pancreatic cancer; differentiation

Exosomal nanoparticles are small vesicles released by many cell types and generated through the fusion of multivesicular bodies with the plasma membrane. These exosomal nanoparticles, also referred to as exosomes, were initially described during maturation of reticulocytes, where they function as garbage bags to discard plasma membrane proteins that are no longer necessary. They also act as messengers in intercellular communication. Since their first description in hematopoietic cells, it has been clearly shown that exosomal nanoparticles are secreted by many non-hematopoietic cell types, such as intestinal epithelial cells,[1] neuroglial cells,[2] and cancer cells from breast,[3] liver,[4] ovary,[5] prostate,[6] and melanoma.[7] Biochemical studies have shown that only a subset of cell macromolecules is shed through the nanoparticle pathway.[8] The biological significance of exosomal nanoparticles is largely questioned.[9] However, the presence of these vesicles in human blood suggests that they have a physiological role in cell-cell or organ-organ communication.[10] Exosomes are believed to play an antitumoral role through immune cells.[8]

We recently showed that human pancreatic tumoral cells expressed exosomal nanoparticles that are rich in lipid-forming microdomains termed rafts.[9,11] These exosomal nanoparticles interact with tumoral cells to activate PTEN and GSK-3β and decreased the mitochondrial pyruvate dehydrogenase (PDH) activity. Moreover, this interaction led to increased Bax and decreased Bcl-2 expressions. Caspase-3 and -9 inhibitors impaired apoptosis, which therefore implicates the mitochondria-dependent apoptotic pathway. Lastly, exosomal nanoparticles triggered the down regulation of cyclin D1 and inactivated PARP. Hence, exosomal nanoparticles counteracted the constitutively activated PI3K/Akt survival pathway to drive tumoral cells towards apoptosis.[11] However, exosomal nanoparticles do not affect the proliferation of endothelial cells of normal human umbilical vein.[11] Thus, we have hypothesized that tumoral cell exosomal nanoparticles may regulate tumor self-growth to favor the establishment of the neovasculature as they bear angiogenic factors.[11]

Putative cell membrane targets of exosomal nanoparticles are lipid rafts.[12] Lipid rafts are platforms for cell signaling,[13] and raft integrity is required in particular for the localization and functioning of growth factor receptor,[14] kinase activation,[15] and secretase-shedding of cell-anchored growth-factor.[16] We showed that the effects of exosomal nanoparticles varied depending on cell lines,[11] possibly according to their differentiation state. In this context, Notch signaling may be a key element as it regulates the balance between self-renewal and differentiation, favoring the expansion of pancreatic cancer cell progenitors.[17,18] Furthermore, β-secretase and γ-secretase that participate in Notch-1 maturation and functioning are integral membrane proteins forming a complex with Notch-1 localized in lipid rafts.[16,19,20] The function of this complex is sensitive to the surrounding lipid environment.[21–23]

The present study aims at elucidating the mechanisms by which exosomal nanoparticles regulated tumoral cell proliferation. We determined that the interaction of exosomal nanoparticles with pancreatic tumoral cells led to decreased expression of Hes-1, the well-known intracellular target of Notch-1. We demonstrated that the expression levels of partners in the Notch-1 pathway such as Hes-1, ICN and ADAM 17, were critical factors leading to tumoral cell sensitivity to exosomal nanoparticles. These levels of expression of Notch pathway components inversely correlated with the differentiation state of tumoral cell lines. Exosomal nanoparticles decreased the proliferation of differentiated tumoral cells but they did not affect that of poorly differentiated cells. Overexpression of Notch-1 intracellular domain resulted in the reversion of the proliferation inhibition promoted by exosomal nanoparticles. The interaction of exosomal nanoparticles with differentiated tumoral cells led to activation of the apoptotic pathway after a cell cycle arrest in G_0G_1 phase.

Abbreviations: ADAM, a disintegrin and metalloproteinase domain; [bpV(phen)], potassium bisperoxo (1,10-phenanthroline) oxovanadate; FCS, fetal calf serum; GSK-3β, glucose synthase kinase-3β; Hes, hairy and enhancer-of-split homolog; ICN, intracytoplasmic Notch; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly ADP-ribose polymerase; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

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Furthermore, ADAM17 was localized in lipid rafts, which are reported to also associate Notch and the γ-secretase complex, all partners most likely involved in the interaction with the exosomal nanoparticles. We finally showed that a cross-talk between Notch-1 signaling pathway and GSK-3β and PTEN took place in pancreatic tumoral cells.

Our study provides evidence that Notch signaling and apoptotic pathways intertwine in tumoral cells in which the expression level of Notch-1 partners determine the sensitivity to exosomal nanoparticles and thus the fate of the tumoral cell.

Material and methods

Materials

Peroxidase-labelled antibodies to rabbit, antibodies to PTEN, to (Ser380)phospho-PTEN, to GSK-3β, to (Ser9)phospho GSK-3β, to cleaved caspase-9 (Asp330), to Bax and to PARP were from Cell Signaling (Beverly, MA). Peroxidase-labelled antibodies to mouse and goat immunoglobulins and antibodies to β-actin were from Sigma (St. Louis, MO). Antibodies to ADAM 17, and to caveolin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Hes-1 and Notch-1 (activated intracytoplasmic Notch form, ICN) were from Abcam (Cambridge, UK). Antibodies to CD9[24] were a kind gift from Dr. E. Rubinstein (INSERM UMR 602, Villejuif, France). RPMI 1640, DMEM media, penicillin, streptomycin, trypsin-EDTA were purchased from Cambrex (Cambrex Biosciences, Emerainville, France). LiCl, L-685,458 and bpV(phen) (potassium bisperoxo (1,10-phenanthroline) oxovanadate) were from Sigma and from Alexis (San Diego, CA), respectively.

Cell growth and apoptosis

Cell lines originating either from human pancreatic carcinoma or adenocarcinoma were grown in RPMI 1640 (BxPC-3 and SOJ-6) or DMEM (MiaPaCa-2, Panc-1 and HEK 293) medium with 10% fetal calf serum (FCS) unless otherwise stated (cells were termed "quiescent" when they were grown in the presence of 0.1% heated FCS or in the absence of FCS). Tumoral cells were seeded in a 96-well culture plates (8,000 cells /well) for 24 h, and treated with increasing amounts of exosomal nanoparticles for another 24 h. Cell growth was assessed by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT was added for the last 4 h of culture. Apoptosis was determined using the CaspACE-FITC-VAD-fmk *in situ* marker (Promega, Madison, WI) as already described.[11] The number of apoptotic fluorescent cells was determined in triplicate on collections of fields randomly examined under a fluorescent microscope (Carl Zeiss, New York, NY).

Cell transfection

Transient transfection of HEK (Human Embryonal Kidney) 293 cells at 50–80% confluence in DMEM (10% FCS) was performed with pEGFP-C1 and pEGFP-NICD plasmids expressing either fluorescent protein EGFP or EGFP and the active intracellular domain of Notch-1 (both plasmids are gifts from Dr. Freddy Radtke, Epalinges, Switzerland) using the LipofectAMINE-mediated transfection kit according to the manufacturer' instructions (Invitrogen). HEK 293 cells were incubated with the transfection medium at 37°C in 5% CO_2 for 6–12 h, then the medium was removed and cells were incubated in 10% FCS-DMEM. Cells were used after 24 h and only when 80–90% transfection was reached with fluorescent EGFP. Cells were then seeded in 96-well culture plate as above and cultured for 24 h in 0.1% FCS DMEM medium. Quiescent HEK 293 cells were challenged with 5 μg/ml exosomal nanoparticles from SOJ-6 cells, and cell growth was finally assessed with MTT.

Flow cytometry analyses

For cell cycle distribution experiments, SOJ-6 cells were incubated with serum-free medium and in the absence (control) or in the presence of nanoparticles (5 μg/ml) for 24 h. Cells were harvested using a non-enzymatic cell dissociation solution (Sigma), centrifugated and resuspended in 70% ethanol, and stored overnight at −20°C. Cells were washed in cold PBS, incubated in RNAse solution for 20 min at room temperature, stained with propidium iodide (100 μg/ml) in Isoflow solution, and left to incubate for 30 min at room temperature. Cell cycle distribution was detected by flow cytometry using FACSCalibur flow cytometer (Beckton-Dickinson) and the fraction of cells in the sub-G_1, G_0G_1, S and G_2M phases was determined using Modfit LT software (Verity Software House, Inc., Topsham, ME). The red fluorescence of single events was recorded using excitation and emission at 488 nm and at 610 nm respectively, to measure the DNA index.

Isolation of raft lipid domains

Raft lipid domains of SOJ-6 cells were isolated by the one-step procedure described by Tellier *et al.*[16] For this purpose cells were washed with ice-cold PBS (without Ca^{++} and Mg^{++}), and then harvested by scraping in PBS. The cells were collected by centrifugation, suspended in 900 μl of 20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA buffer with protease, phosphatase and metalloproteinase inhibitors, snapped frozen in liquid nitrogen and thawed on ice. The cell suspension was passed 10 times through a 25-gauge needle. Lysis with Brij98 was performed by equilibrating the cell suspension at 37°C, followed by the addition of Brij 98 at 1% final concentration (w/v). The Brij 98 cell lysate was then centrifuged (1,000*g*, 10 min, 4°C) to remove nuclei and cellular debris. The resulting supernatant was submitted to high-speed centrifugation (100,000*g*, 1 h, 4°C) to pellet the Brij 98 insoluble material corresponding to the total lipid raft fraction. The pellet was washed with Hepes buffer, then solubilized in SDS-PAGE sample buffer for Western-blot analyses.

Exosomal nanoparticle purification

Pancreatic cells were grown up to 80% confluence in appropriate medium with 10% FCS. Then the medium was removed, cells rinsed 3 times with PBS and maintained for 24 h in a serum-free medium (13 ml for 20 cm diameter dish). During this last step, no loss of cell viability was recorded as estimated by a trypan blue test. The supernatant was recovered and centrifuged at 4°C for 20 min at low speed (1,000*g*) to remove cell debris before being percolated through out 0.22 μm filters. Finally, the cleared medium was submitted to an ultracentrifugation at 200,000*g* for 16 h at 4°C by using SW41 swinging bucket centrifuge rotor (Beckman Coulter, Roissy, France). The final pellet, containing exosomal nanoparticles, was resuspended in 0.5 ml PBS for further analyses. An aliquot was saved for protein determination using the Bradford's assay instead of the bicinchoninic acid assay previously used[11] and leading to erroneously high values for proteins in the presence of phospholipids.[25]

SDS-PAGE and western blotting

SOJ-6 cells were grown in 6-well culture plates in RPMI 1640 medium with 10% FCS. At subconfluency, the medium was removed and replaced for 24 h by fresh RPMI medium without FCS. Quiescent SOJ-6 cells were then incubated with exosomal nanoparticles (5 μg/ml) for various times. Alternatively SOJ-6 cells were incubated with drugs (LiCl, bpV(phen)] or L-685,458) up to 48 h in RPMI medium without FCS. After treatment, the cells were washed 3 times with ice-cold PBS (without Ca^{++} and Mg^{++}), harvested with a rubber policeman and pelleted by centrifugation. Pellets were washed twice and lysed at 4°C in 0.5 ml of lysis buffer (10 mM Hepes pH 7.4, 200 mM NaCl, 1.5% Triton X-100, 5 mM EDTA, 2.5 mM $MgCl_2$, and 2 mM $CaCl_2$, protease inhibitors (Complete TM, Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktail (Sigma). After

lysis, homogenates were sonicated (10 sec, 40 W, 4°C) clarified by centrifugation (10,000*g*, 15 min, 4°C). An aliquot was saved for protein determination using the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins in reducing SDS buffer were separated on polyacrylamide gels (from 7.5 to 12% according to the molecular weight of the protein to be separated) and 0.1% SDS. After electrophoretic migration, proteins were transferred onto nitrocellulose membranes using a Mini Transblot electrophoretic cell (BioRad). Transferred proteins were immunodetected by using appropriate primary and secondary antibodies. After washes, membranes were developed with a chemoluminescent substrate according to manufacturer's instructions (Roche Diagnostics). In each experiment, a control was included by omitting primary antibodies or by using non-immune serum.

Reverse transcription and polymerase chain reaction

The cells were washed thrice in ice-cold PBS (without Ca^{++} and Mg^{++}) and total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions and precipitated in isopropanol. RNA samples were treated with DNase (RQ1 RNAse-free DNAse, Promega) to remove traces of contaminant genomic DNA. RNA integrity was checked over on an agarose gel and concentration was determined by optical density. Reverse transcription (RT) reactions were performed on 5 μg of total RNA using random hexamers and the M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Primers were designed to amplify a 400–1,200 bp fragment in the coding sequence of genes. Nucleotide sequences were aligned using the MultAlin software in order to design specific primers. The house keeping gene encoding GAPDH was chosen as control. PCR was performed on 5 μl of template cDNA in a 50-μl final volume containing 0.5 μM of each forward and reverse primer and the PCR mix for GoTaq (Promega) according to the manufacturer's instructions. PCR cycles were : denaturation ; 1 cycle 5 min, 94°C, followed by 35 cycles: denaturation ; 1.5 min, 94°C, annealing ; 1.5 min at T_m, extention ; 2 min, 72°C and termination 10 min at 72°C. Amplification products were analyzed on agarose gels stained with ethidium bromide. Some specific PCR products were purified from agarose gel using the PCR Clean-up Gel Extraction kit (Macherey-Nagel) and sequenced by Genome Express (Meylan, France). Note that PTEN and GSK-3β primers allow the amplification of the full size cDNA for complete sequencing and mutation record. Primers were as follows : Forward PTEN-5′-HindIII: 5′TTTCGTAAGCTTATGACAGCCATC ATCAAA3′, Reverse PTEN-3′-EcoRI: 5′TTTCGTGAATTCTCA GACTTTTGTAATTTGTGT3′ (1.2 kb, T_m = 57°C); Forward GSK-3β-5′-HindIII: 5′TTTCGTAAGCTTATGTCAGGGCGGCC CAG3′; Reverse GSK-3β-3′-EcoRI: 5′TTTCGTGAATTCTCAG GTGGAGTTGGAAGCTGA3′ (1.2 kb, T_m = 65°C); Forward Hes-1: 5′ACACCGGATAAACCAAAGAC3′; Reverse Hes-1: 5′TGATCTGGGTCATGCAGTTG3′ (0.4 kb, T_m = 60°C); Forward ADAM 17: 5′GCATTCTCAAGTCTCCACAAG3′; Reverse ADAM 17: 5′CCTCATTCGGGCACATT CTG3′ (0.4 kb, T_m = 55°C); Forward GAPDH: 5′GGGAAGGTGAAGGTCGGAG3′; Reverse GAPDH: 5′ GAGGGGGCAGAGATGATGA3′ (0.4 kb, T_m = 60°C).

Statistical analysis

Each experiment was done at least 3 times. When indicated, results were expressed as means ± SD. Differences between experimental groups were analyzed with the Mann-Whitney test. A value of $p < 0.05$ was considered significant.

Results

Exosomal nanoparticles decreased growth of human cells from differentiated pancreatic adenocarcinoma

We have previously documented that exosomal nanoparticles purified from SOJ-6 cells induce apoptosis of various human pancreatic tumoral cell lines.[11] Here, we first investigated the ability of exosomal nanoparticles from each of these lines to decrease cell growth. Human pancreatic (adeno)carcinoma cells, either differentiated SOJ-6 and BxPC-3 cell lines or poorly differentiated MiaPaCa-2 and Panc-1 cell lines,[26,27] were incubated for 24 h with increasing amounts of freshly purified exosomal nanoparticles (from 0.5 μg/ml up to 10 μg/ml in term of proteins) isolated from SOJ-6, BxPC-3, MiaPaCa-2, or Panc-1 cells. Cell proliferation was then monitored using MTT assay. Cell treatment with exosomal nanoparticles from SOJ-6 and BxPC-3 cells resulted in a dose-dependent inhibition in cell proliferation (Figs. 1*a* and 1*b*) whereas exosomal nanoparticles from MiaPaCa-2 and Panc-1 cells were quite inefficient (Figs. 1*c* and 1*d*). Significant decreases in proliferation rates of SOJ-6 and BxPC-3 cells, compared to those of controls without exosomal nanoparticles ($p < 0.05$), were obtained with exosomal nanoparticles from either SOJ-6 or BxPC-3 cells (Figs. 1*a* and 1*b*). The most important inhibitory effects on proliferation (about 40–45% inhibition) were observed with 10 μg/ml exosomal nanoparticles. MiaPaCa-2 cells, which derived from poorly differentiated carcinoma, produced exosomal nanoparticles that lacked the ability to inhibit tumoral cell growth. MiaPaCa-2 cells were poorly sensitive to exosomal nanoparticles from differentiated cells, BxPC-3 and SOJ-6.

Exosomal nanoparticles decreased the expression of the Notch signaling partner Hes-1 and activated the mitochondria-dependent apoptotic pathway in SOJ-6 cells

Our previous study suggested that raft microdomains in tumoral plasma membrane may be cell targets for exosomal nanoparticles, which leads to activation of the mitochondria apoptotic pathway.[11] Besides, it is also known that ADAM 17, γ-secretase and Notch-1, which are all linked to Notch-1 signaling, are mainly sequestered in lipid rafts.[16,19,20] We firstly ensured that ADAM17 was localized in our preparation of membranes, enriched in caveolin that most likely are raft lipid domains[16] (Fig. 2*a*). ADAM 17 could be hardly detected in the SOJ-6 whole cell lysate possibly due to its low expression level. Nevertheless, the overexposition of the film made the detection of ADAM 17 possible in SOJ-6 cell lysates (Fig. 2*a*, lower panel). Furthermore, ADAM 17 was detected in raft membrane domains of SOJ-6 cells. Challenging these cells with exosomal nanoparticles did not modify the ADAM 17 localization in rafts (Fig. 2*a*). Yet, it revealed a slight apparent increase in ADAM 17 in raft fraction upon exosomal nanoparticles cell challenging, which was unlikely due to exogenous protein, as ADAM 17 was not detected in exosomal nanoparticles, in contrast to CD9, a well-known exosome marker (Fig. 2*a*).

Next, the fact that the Notch-1 pathway is a major survival pathway in pancreatic cancer[28] prompted us to ask whether exosomal nanoparticles could intertwine with Notch-1 signaling. We therefore examined the effects of exosomal nanoparticles on Hes-1 expression, which is an intranuclear end-product in the Notch-1 signaling pathway. Figure 2*b* showed that Hes-1 expression is significantly decreased by 21 and 64% upon SOJ-6 cells challenging with exosomal nanoparticles for 24 and 48 h, respectively. The decrease in Hes-1 expression strongly suggested that the Notch-1 signaling pathway is a target of these exosomal nanoparticles.

It is known that the down-regulation of Notch-1 and the decrease in Hes-1 expression contribute to pancreatic cancer cells apoptosis.[18] Flow cytometry analyses (Fig. 2*c*) indicated that the amount of cells in the G_0G_1 phase was significantly increased by the treatment of cells with exosomal nanoparticles ($p < 0.05$ when compared to untreated control cells). However, the S and the G_2M, cell phase fractions were significantly decreased in an exosomal nanoparticle concentration manner. Consequently, the sub-G_1 fraction containing apoptotic cells was significantly increased from approximately 4% in untreated control cells up to 16% ($p < 0.01$) in cells upon treatment with increasing concentrations of exosomal nanoparticles. These data suggested that exosomal nanoparticles promoted a blockade of SOJ-6 cells in the G_1 phase that evoked apoptosis. Therefore, apoptosis was further

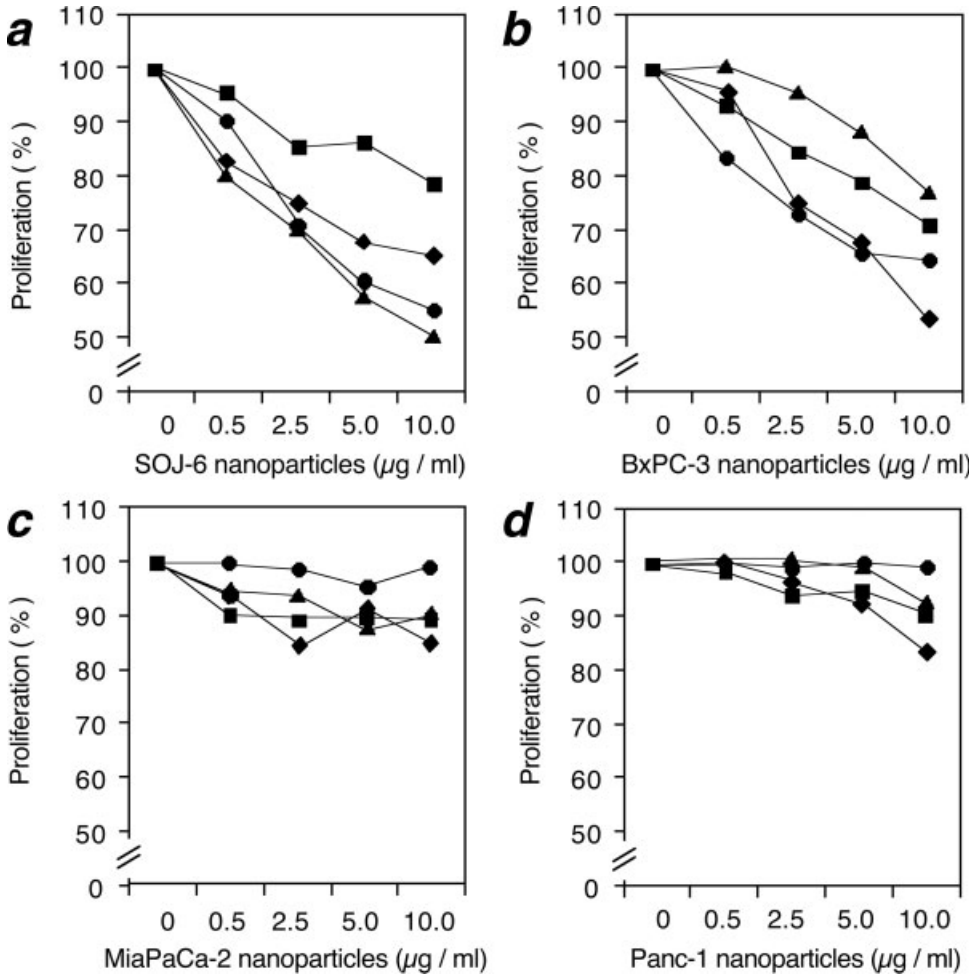


Figure 1 – Proliferation of SOJ-6, BxPC-3, MiaPaCa-2 and Panc-1 human pancreatic cell lines in the presence of exosomal nanoparticles. Quiescent cells were incubated with increasing concentrations (up to 10 μg/ml) of exosomal nanoparticles isolated from culture medium of SOJ-6 (*a*), of BxPC-3 (*b*), of MiaPaCa-2 (*c*), and of Panc-1 (*d*) cells. Cells from the 4 pancreatic tumoral lines, SOJ-6 (circles), BxPC-3 (diamonds), MiaPaCa-2 (squares), and Panc-1 (triangles), were challenged with exosomal nanoparticles from each cell line origin. After 24 h of incubation, proliferation was assessed by MTT assay. Results are expressed in percent relative to controls (no added exosomal nanoparticles) taken as 100%. Data are means ± SD of at least 3 independent experiments. Errors are within 10% of experimental values.

determined by Western blotting using specific antibodies to the pro-apoptotic Bax, to cleaved caspase 9, and to PARP. Figure 2*d* indicates that Bax and cleaved caspase 9 accumulated with time in lysates of cells challenged with exosomal nanoparticles. Furthermore, PARP was also cleaved to lead to an inactive 89 kDa fragment. These data provided evidence that the activation of the mitochondria-dependent apoptotic pathway by exosomal nanoparticles concomitantly took place with decreased Hes-1 expression.

Expression levels of Notch-1 pathway partners depended on the differentiation state of pancreatic tumoral cell lines

Since poorly differentiated tumor cells were less sensitive than differentiated ones to apoptotic exosomal nanoparticles, we thought to study a possible relationship between the expression level of Notch-1 partners in all tumoral cell lines and the sensitivity of these cell lines to exosomal nanoparticles. Knowing that (*i*) ADAM 17 and Hes-1 expression are linked to cell differentiation in Notch-1 signaling,[29,30] (*ii*) Notch-1 is overexpressed in pancreatic cancer[28] and (*iii*) sensitive cells (SOJ-6 and BxPC-3) and poorly sensitive cells (MiaPaCa-2 and Panc-1) originated from a differentiated and from poorly differentiated tumors respectively,[26,27] we first examined Notch-1 partner transcripts in the 4 tumoral cell lines. RT-PCR experiment showed that ADAM 17 and Hes-1 are encoded in each cell line (Fig. 3*a*). However, the analysis of lysates of the various tumoral cells by Western blotting indicated that the amounts of ADAM 17, Hes-1, and intracytoplasmic Notch (ICN) are higher in poorly differentiated cells (MiaPaCa-2 and Panc-1) than in sensitive differentiated SOJ-6 and BxPC-3 cells (Fig. 3*b*). These data suggested that ADAM17 and Hes-1 expressions were post-transcriptionally regulated, which is in line with recent findings.[31,32] Thus, the less Notch-1 partners were expressed, the more the cells were sensitive to exosomal nanoparticles. To confirm this hypothesis, we transfected cells with the plasmid encoding for ICN, a dominant active receptor independent of ligand. Because the levels of transfection of SOJ-6 and of BxPc-3 pancreatic cells with the pEGFP-NICD plasmid encoding ICN remained low, we transfected another cell line, the HEK (human embryonal kidney) 293 cells. Its transient transfection reached 80–90% as shown by EGFP fluorescence. The Western blotting (Fig. 3*c*, upper panel) indicated that HEK 293 cells transfected with pEGFP-NICD (ICN+) overexpressed ICN when compared to parental HEK 293 cells (WT) and to pEGFP-C1 control vector-transfected cells (ICN-). As shown on Fig. 3*c* (lower panel) HEK 293 cells transfected with the control vector were significantly sensitive to exosomal nanoparticles from SOJ-6 cells. In contrast, the cells overexpressing ICN become insensitive to exosomal nanoparticles as their proliferation level reached that of

control. Consequently Notch-1 appeared to be a cell target of exosomal nanoparticles.

Gamma-secretase inhibitor L-685,458 made poorly differentiated tumoral MiaPaCa-2 cells sensitive to exosomal nanoparticles

Collectively, the above data supported the hypothesis that the constitutive expression level of Notch-1 signaling partners could be implicated in the cell sensitivity to exosomal nanoparticles. To further ascertain the involvement of the Notch-1 signaling pathway in exosomal nanovesicle effects on pancreatic tumoral cells, we used L-685,458, a specific inhibitor of presenilin, a key-element of the γ-secretase complex involved in the signaling *via* Notch-1.[33] Strikingly, as shown in Figure 4*a* (upper panel) MiaPaCa-2 cells, which are poorly sensitive to MiaPaCa-2 exosomal nanoparticles (Fig. 1*c*) and to L-685,458 became highly sensitive to their own exosomal nanoparticles (5 μg/ml) in the presence of the presenilin inhibitor (5 μM), which was assessed by a marked decreased proliferation (upper panel, left) and by a clearly increased number of apoptotic cells (Fig. 4*a*, upper panel, right). This result supports the contention that decreasing the level of active γ-secretase in the Notch-1 pathway of poorly sensitive cells was sufficient to make these cells highly sensitive to exosomal nanoparticles.

In contrast, L-685,458, at the used concentrations in the absence of exosomal nanoparticles, decreased the proliferation of highly sensitive SOJ-6 cells and induced apoptosis in a dose-dependent manner (Fig. 4*a*, left and right lower panel). These results mean that the inhibition of the Notch-1 pathway evoked pancreatic cancer cells apoptosis as already observed.[18] Slopes depicting decreases in cell proliferation are identical independently of the presence of exosomal nanoparticles meaning that the effects of presenilin inhibitor were additive to that promoted by exosomal nanoparticles (used at concentration promoting partial cell proliferation inhibition).

Gamma-secretase inhibitor L-685,458 led to Hes-1 down-regulation and to PTEN and GSK-3β activation

To explore biological consequences of inhibiting γ-secretase complex, we used L-685,458. As shown on Figure 4*b*, challenging SOJ-6 cells with increasing concentrations of L-685,458 decreased Hes-1 expression, as exosomal nanoparticles did (Fig. 2*b*). Since Hes-1 was recently shown to negatively control the expression of PTEN,[34] we looked at the expression of PTEN in both dephosphorylated and phosphorylated states. Figure 4*b* clearly demonstrated that challenging SOJ-6 cells with increasing concentrations of L-685,458 also led to decreased PTEN and GSK-3β phosphorylation

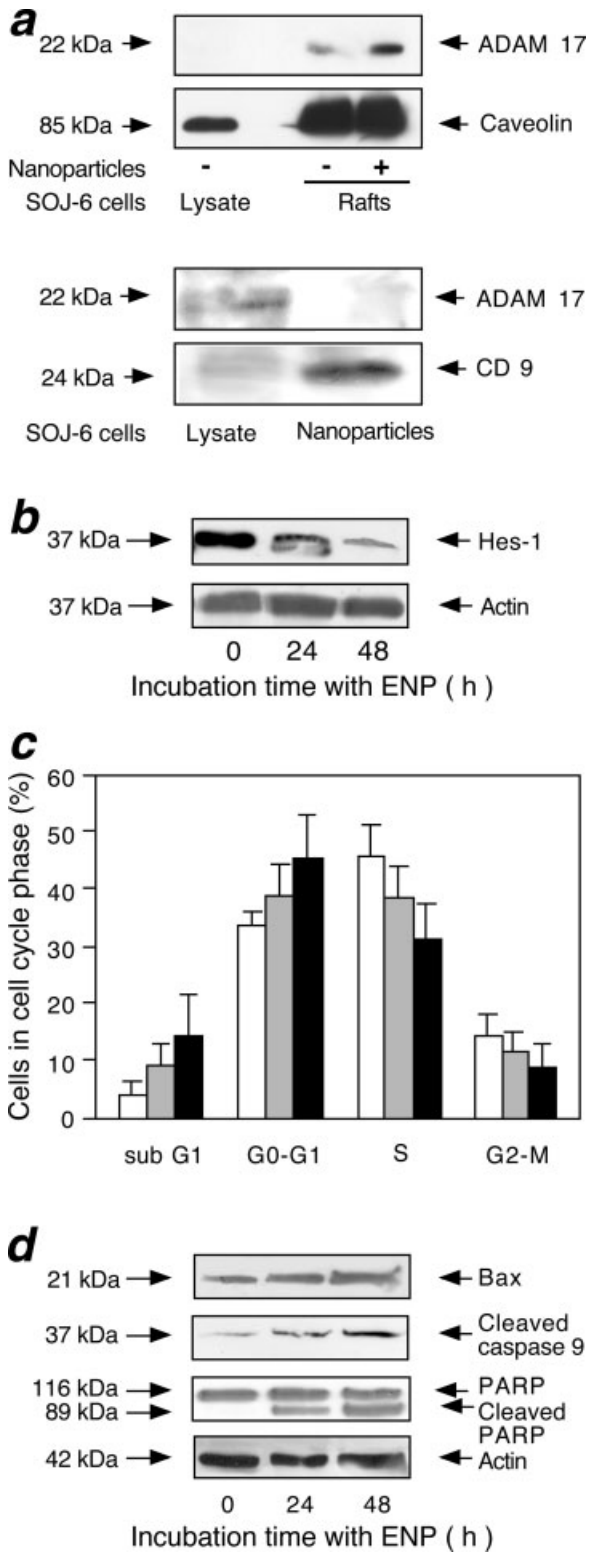
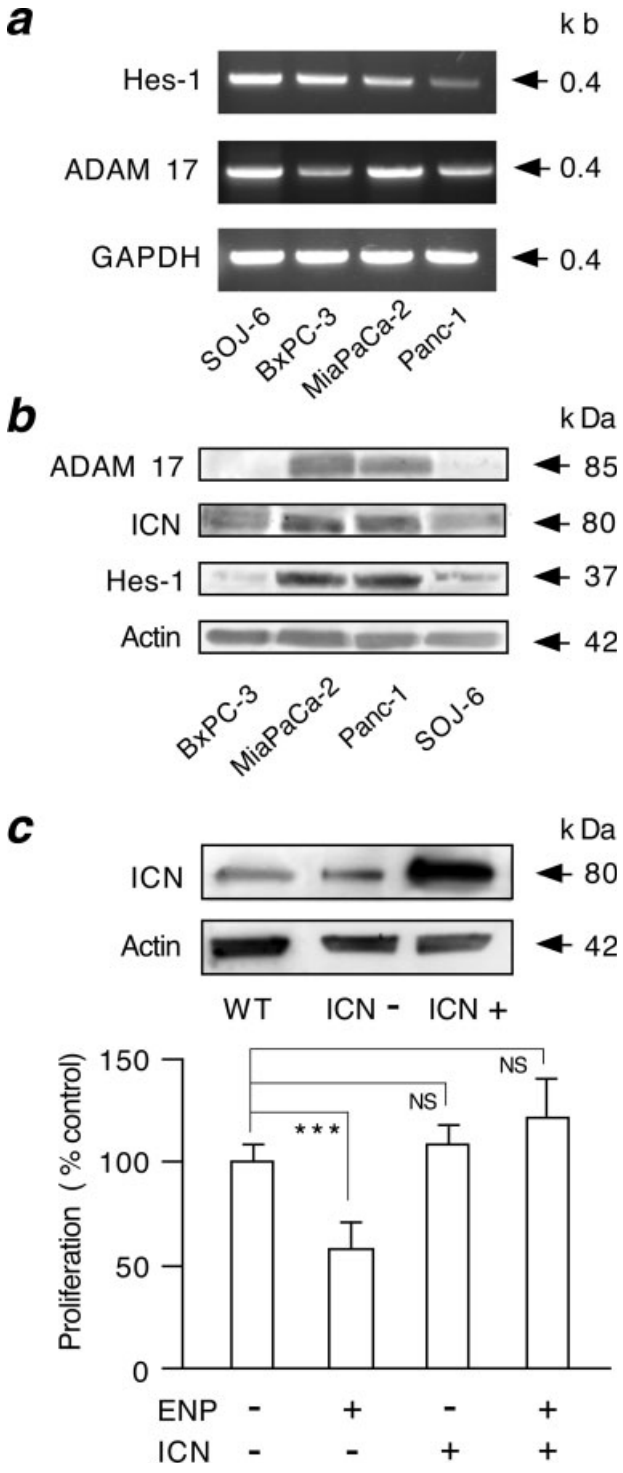


FIGURE 2 – Effects of exosomal nanoparticles on raft localization of ADAM 17, and on the expression of Hes-1 and of components of the mitochondria-dependent apoptotic pathway. (*a*) Raft lipid domains and lysates of SOJ-6 cells, either incubated (+) or not (−) with exosomal nanoparticles from SOJ-6 cells, were isolated from the same number of cells by the one-step procedure described by Tellier *et al*.[16] and analyzed by Western blotting using antibodies to ADAM 17 and to caveolin (upper panel). Western blottings were also performed on lysate of SOJ-6 cells and on nanoparticles isolated from SOJ-6 cells (50 μg proteins per lane) using antibodies to ADAM 17 and to CD 9 (lower panel). (*b*) Quiescent SOJ-6 cells were challenged for 0, 24 or 48 h with exosomal nanoparticles (5 μg/ml). Proteins from cell lysate were separated on SDS-PAGE and electrotransferred onto nitrocellulose membrane. Hes-1 expression was detected by Western blotting using specific antibodies. The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing. (*c*) Cell cycle distribution was examined by propidium iodide staining and flow cytometry on SOJ-6 cells challenged for 24 h with 0 (control, open column), 2.5 (shaded column) and 5 μg/ml (dark column) exosomal nanoparticles isolated from SOJ-6 cells. Data are means ± SD of 3 independent experiments. (*d*) SOJ-6 cells were treated as in (*b*) and cell lysate proteins were separated. The levels of Bax, (cleaved)PARP and cleaved caspase-9 were determined by probing membranes with antibodies specific to each protein. Lysates of SOJ-6 cells, challenged for 0, 24 or 48 h with exosomal nanoparticles (5 μg/ml), were used for Western blottings. All the western blotting experiments were done in triplicate and one representative experiment is shown here. Control experiments in (*b*) and (*d*) were performed with cells incubated for 48 h without exosomal nanoparticles (ENP) whose results were similar to that obtained at time 0 with ENP. The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing.

at Ser380 and at Ser9, respectively. Such a decrease in PTEN and GSK-3β phosphorylation could not be seen by Western blotting analysis in poorly sensitive MiaPaCa-2 cells. This corroborated the low level of apoptosis of MiaPaCa-2 cells upon incubation with the γ-secretase inhibitor (Fig. 4*a*, upper panel). Also the expression of Hes-1 was not affected by exosomal nanoparticles treatment of the latter cells (Fig. 4*b*). The phosphorylation of PTEN and of GSK-3β observed in the absence of the drug confirmed that the PI3K/Akt pathway is constitutively active in SOJ-6 as well as in MiaPaCa-2 cells, as already observed.[11]



Also, knowing that the preponderance of apoptosis induced by exosomal nanoparticles over survival results particularly from the activation of GSK-3β and PTEN,[11] we compared the expression of PTEN in all pancreatic cell lines used here. Of note, PTEN-deficient cells are insensitive to exosomal nanoparticles, and inhibiting PTEN in PTEN-wild type cells decreased the sensitivity of cells to exosomal nanoparticles. PTEN is rarely mutated in pancreatic adenocarcinoma.[35] Moreover it is known that PTEN cDNA is not mutated in SOJ-6, BxPC-3 and Panc-1 cells[11,36,37] or in MiaPaCa-2 cells (data not shown). Therefore, mutation leading to the functional loss of PTEN cannot account for differences in sensitivity to exosomal nanoparticles. We also checked that GSK-3β was not mutated in SOJ-6 and in MiaPaCa-2 cells (data not shown).

Figure 4*c* indicated that similar levels of PTEN transcript were detected in each cell line. GSK-3β, which forms a complex with PTEN upon cell challenging with exosomal nanoparticles,[11] was also encoded to similar levels in each cell line (Fig. 4*c*). Western blots using specific antibodies to PTEN and GSK-3β confirmed the expression of these two proteins in each pancreatic cell line. Moreover the amounts of PTEN and of GSK-3β did not differ from cell to cell (Fig. 4*d*). We therefore concluded that differences in sensitivities of pancreatic tumoral cell lines to exosomal nanoparticles did not depend on the PTEN or GSK-3β functionality and expression level.

PTEN and GSK-3β inhibition led to Hes-1 up-regulation and counteracted the exosomal nanoparticle - induced inhibition of proliferation

Because inhibiting γ-secretase led to Hes-1 down-regulation and to PTEN and GSK-3β activation (Fig. 4*b*), we hypothesized that PTEN and/or GSK-3β activities in turn may down-regulate Hes-1 expression. We thus investigated the effects of specific inhibitors of PTEN and of GSK-3β, bpV(phen) and LiCl respectively on Hes-1 expression. As shown on Figure 5*a*, inhibiting either PTEN or GSK-3β activities led to Hes-1 up-regulation in both cases, which correlated with the reversion of cell proliferation inhibition promoted by exosomal nanoparticles (Fig. 5*b*). Interestingly, the effects of these 2 inhibitors were not additive on the reversion process, suggesting that these 2 proteins could be functionally linked. To address this question, we evaluated the phosphorylation of GSK-3β and PTEN in the presence of LiCl and bpV(phen). As shown in Figure 5*c*, LiCl, the GSK-3β inhibitor, did not affect PTEN phosphorylation. In contrast, inhibiting PTEN with bpV(phen) led to increased phosphorylation of GSK-3β. This indicates that GSK-3β could be a substrate for PTEN protein phosphatase activity. Our data clearly showed that there is

Figure 3 – Expression of Notch-1 pathway partners in cell lines. (*a*) RT-PCR experiments using primers specific of indicated genes were performed on RNA isolated from each pancreatic cell line as mentioned. GADPH levels were used as internal controls. (*b*) Cell lysate proteins of each pancreatic line were separated on SDS-PAGE and were analyzed by Western blotting using primary antibodies as indicated. Each lane was loaded with 50 μg of proteins and β-actin was used as loading control. (*c*) Transient transfection of HEK cells with the Notch-1 intracellular domain (ICN). HEK 293 cells were transiently transfected with the pEGFP-C1 (ICN−) and pEGFP-NICD (ICN+) plasmids. Quiescent transfected HEK 293 cells were challenged without (ENP−) or with 5 μg/ml (ENP+) exosomal nanoparticles from SOJ-6 cells for 24 h. Cell lysate proteins of parental and transfected HEK 293 cells were separated on SDS-PAGE and analyzed by Western blotting using primary antibodies as indicated. Western blottings (upper panel) depicted the expression level of ICN in parental (WT), pEGFP-C1 control vector-transfected (ICN−) and pEGFP-NICD-transfected (ICN+) HEK 293 cells cultured in DMEM with 10 % FCS. The amount of loaded proteins was the same for each lane (25 μg) as indicated by β-actin probing. Cell growth (lower panel) was determined with MTT. The proliferation of HEK 293 cells transfected with the control vector (ICN−) recorded in the absence of exosomal nanoparticles (ENP−) was taken as 100%. Values are means ± SD of at least 3 independent experiments (***$p < 0.001$).

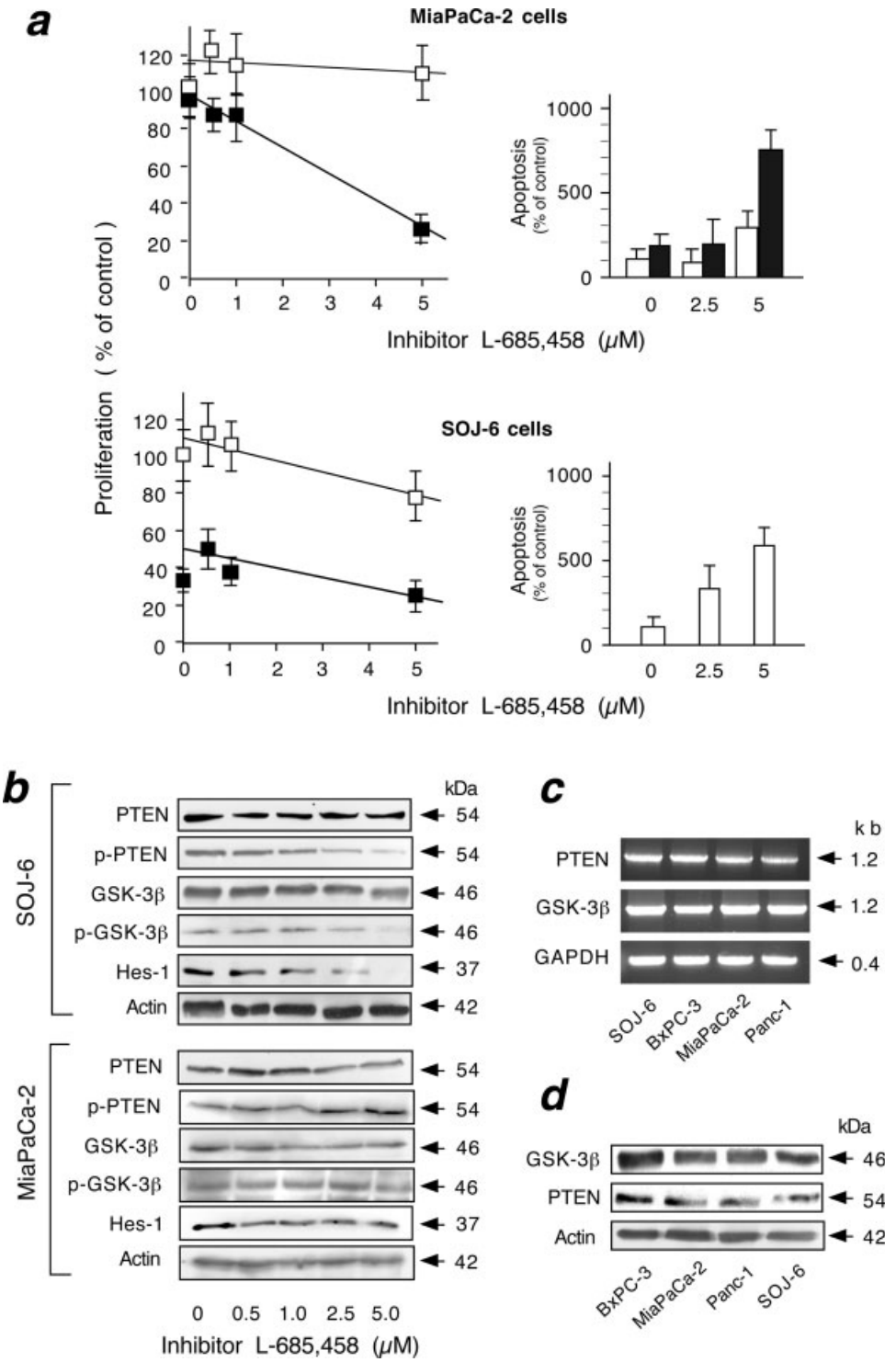


Figure 4 – Effects of the gamma-secretase inhibitor L-685,458 on tumoral cell sensitivity to exosomal nanoparticles, and expression of GSK-3β and of PTEN by tumoral pancreatic cells. (*a*) MiaPaCa-2 (upper panel) and SOJ-6 (lower panel) cells were incubated up to 80% confluence in 96-well plates. Cells were incubated in the presence of L-685,458 γ-secretase inhibitor, at the indicated concentrations, in the absence (open symbols) or presence (full symbols) of 5 μg/ml of their respective exosomal nanoparticles. After 24 h of incubation, cell proliferation was assessed by MTT assay. Cell apoptosis was recorded using the CaspACE-VAD-fmk *in situ* marker (cells without exosomal nanoparticle, open column; cells with 5 μg/ml exosomal nanoparticles, dark column). Results represent the percent of apoptotic fluorescent cells in randomly-analyzed microscope fields relative to control values taken as 100%. Values are means ± SD of 3 independent experiments. (*b*) SOJ-6 and MiaPaCa-2 cells were cultured to 80% confluence in 6-well plates and further incubated for 24 h with increasing concentrations of L-685,458 in serum-free medium. At the end of the incubation, cells were washed, harvested, and lysed in lysis buffer including inhibitors of phosphatases and of proteases. Cell lysate proteins were separated on SDS-PAGE and analyzed by Western blots using specific antibodies as mentioned. The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing. (*c*) RT-PCR experiments using primers specific of indicated genes were performed on RNA isolated from each pancreatic cell line as mentioned. GADPH levels were used as internal controls. (*d*) Cell lysate proteins of each pancreatic line were analyzed by Western blotting using specific antibodies. The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing.

a cross-talk between some partners of Notch-1 signaling pathway and PTEN and GSK-3β.

Discussion

In a recent report we showed that tumoral exosomal nanoparticles, rich in lipid rafts, from human pancreatic cell lines, decreased tumoral cell proliferation through mitochondria-dependent apoptosis.[11] The plasmalemma target of exosomal nanoparticles was unknown although the involvement of membrane structures such as raft lipid domains was suspected. In this study, our findings provide evidence for an inverse correlation between cell susceptibility to apoptosis induced by exosomal nanoparticles and cell expression levels of ADAM17, ICN and Hes-1, all partners of the Notch-1 survival pathway. Exosomal nanoparticles decreased Hes-1 expression of the differentiated tumoral pancreatic cells, in which the apoptotic issue is favored. In contrast, exosomal nanoparticles were inefficient on poorly differentiated tumoral cells expressing high levels of Hes-1. Yet, inhibiting the γ-secretase complex decreased Hes-1, and activated PTEN and GSK-3β, as exosomal nanoparticles do in both cases, which restored the susceptibility of these tumoral cells to nanoparticle effects.

Because Notch-1, ADAM17 and the γ-secretase complex are localized in raft lipid microdomains,[16,19,20] (this study as to ADAM17), it is therefore tempting to suggest that these domains are particularly sensitive to exosomal nanoparticles. Presenilin contributes to the catalytic site of γ-secretase that releases ICN due to an unusual intramembrane cleaving of Notch-1.[38] In line with this finding, the activities of the γ-secretase and of the β-secretase are shown to be directly and potently affected by their lipid microenvironment in particular by the levels of cholesterol.[21–23] Furthermore, lipids of exosomal nanoparticles are likely key-components in the inhibition of the proliferation of target cells. The crucial role of lipids is also underlined by the fact that after trypsin treatment or heating, nanoparticles are still able to functionally interact with tumoral cells.[11] Interaction between tumoral cell membranes and exosomal nanoparticles thus must be compatible with the biochemical properties of the latters. It is therefore reasonable to assume that such interactions do not happen at random but are rather restricted to adequate areas of plasma membrane, which allow lipidic interactions. Consequently, we suggest that exosomal nanoparticles that are rich in lipid forming rafts may fuse with cell target possibly at the level of raft microdomains to perturb the pivotal action of the Notch-1 survival pathway of pancreatic tumoral cells.

We report that the expression levels of partners in the Notch-1 survival pathway are a critical factor leading to tumoral cell sensitivity to exosomal nanoparticles. Moreover, we showed that the decrease in cell proliferation mediated by exosomal nanoparticles depended on the differentiation state of cells. Using BxPC-3 and SOJ-6 cells, which originate from well or moderately differentiated adenocarcinoma[26,27] and Panc-1 and MiaPaCa-2, which are from poorly differentiated carcinoma,[27] we found that poorly differentiated cells were much less sensitive to exosomal nanoparticles than the differentiated ones. Thus this sensitivity inversely correlates with the expression levels of ADAM 17/Notch-1 and of downstream partners such as ICN and Hes-1, which are higher in poorly differentiated than in differentiated cell lines. These findings support the idea that cells resistant to exosomal nanoparticles are prone to survival *via* the Notch-1 pathway whereas this

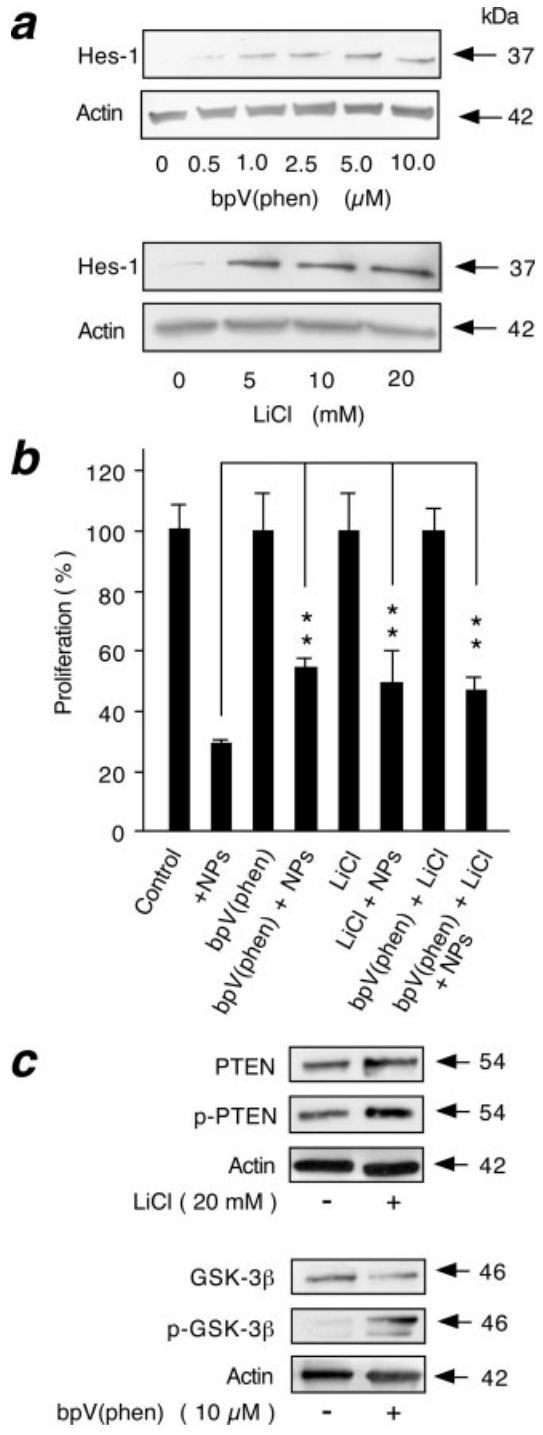


Figure 5 – Effects of PTEN and of GSK-3β inhibition. (*a*) Effects on Hes-1 expression. SOJ-6 cells were cultured to 80% confluence and incubated in serum-free medium in the presence of LiCl or bpV(phen) as specific inhibitors of GSK-3 β and PTEN respectively, at indicated concentrations for 24 h. Cell lysate proteins were separated on SDS-PAGE and analyzed by Western blotting, probed with antibodies to Hes-1. The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing. (*b*) Effects on tumoral cell proliferation. SOJ-6 cells were cultured in 96-well plates and incubated 1 h in the presence of LiCl (5 mM) or bpV(phen) (5 μM) or both without (control) or with exosomal nanoparticles (5 μg/ml, 100 μl/well) for 24 h. At the end of incubation, proliferation was assessed by MTT assay and expressed as % of control. Data are means ± SD of 3 independent experiments (**$p < 0.01$). (*c*) Effects on phosphorylation of PTEN and GSK-3β. SOJ-6 cells were treated as in (*a*). Cell lysate proteins were separated on SDS-PAGE and analyzed by Western blotting, probed with antibodies to PTEN and to (Ser380) phosphorylated PTEN (p-PTEN) or GSK-3β and (Ser9) phosphorylated GSK-3β (p-GSK-3β). The amount of loaded proteins was the same for each lane (50 μg) as indicated by β-actin probing.

pathway may be less efficient in sensitive cells. In contrast, the cell from which the nanoparticles originate may be of importance; for example, SOJ-6 cells and BxPC-3 cells are sensitive to their own exosomal nanoparticles but not to those originating from MiaPaCa-2 and Panc-1 cells. The lipid composition of exosomal nanoparticles is likely important for optimal interaction with cell membranes. Further studies are needed to clarify this specific point. Thus it is reasonable to suppose that responses to exosomal nanoparticles depend at least on 2 factors that are the close interaction between (or fusion of) exosomal nanoparticles and (with) raft microdomains in cell membrane and the efficiency of Notch-1 survival pathway to overcome apoptosis induced by exosomal nanoparticles.

Exosomal nanoparticles failed to significantly decrease the proliferation and to induce apoptosis of poorly differentiated MiaPaCa-2 cells, but inhibiting the γ-secretase by L-685,458, a specific inhibitor of presenilin in the γ-secretase complex, made these cells sensitive to their own exosomal nanoparticles, assessed by both cell proliferation and apoptosis. This reinforces the idea that the level of expression of the partners involved in Notch-1 signaling is crucial, as exosomal nanoparticles decrease the proliferation and induce apoptosis of cells in which these partners are either weakly represented or inhibited (by L-685,458 for example). Furthermore, L-685,458, *per se,* impacts on SOJ-6 cell proliferation and apoptosis. Its effects are additive to that of exosomal nanoparticles suggesting that they impacted on the same target.

Importantly, in SOJ-6 cells, exosomal nanoparticles led to decreased expression of Hes-1, the intracellular target of the Notch-1 signaling pathway and to concomitant activation of the apoptotic pathway. In the same tumoral cells, the inhibitor of presenilin L-685,458 remarkably mimicked the effects of exosomal nanoparticles by promoting apoptosis, by repressing the expression of Hes-1 and by inducing the activation of PTEN and GSK-3β *i.e.*, their dephosphorylation at Ser380 and at Ser9, respectively. Although the γ-secretase regulates a plethora of biological processes,[39] these results demonstrate that the γ-secretase complex plays a crucial role in pancreatic tumoral cell survival. This occurs in a context of (*a*) signaling pathway(s) implicated in the exosomal nanoparticles interaction. This pathway is most likely the Notch-1 survival pathway because (*i*) exosomal nanoparticles reduce Hes-1 expression, which is an intranuclear target of Notch-1, (*ii*) the level of expression of components of Notch-1 signaling pathway is inversely correlated to the effects of nanoparticles, (*iii*) the overexpression of the Notch-1 intracellular domain, ICN, reversed the exosomal nanoparticle-inhibiting effects on exosomal nanoparticle-sensitive cells, (*iv*) ADAM17, ICN and Hes-1 are localized in lipid rafts[16,19,20] where the interaction between exosomal nanoparticles and cell membrane is thought to occur, (*v*) blocking the end step of Notch-1 signaling by an inhibitor of γ-secretase made tumoral cells sensitive to exosomal nanoparticle assessed by inhibition of cell proliferation and apoptosis. (*vi*) in the absence of nanoparticles, the inhibitor of γ-secretase *per se* induces apoptosis in sensitive SOJ-6 tumoral cells. (*vii*) lastly, exosomal nanoparticles provoked the cell cycle arrest in the G_0G_1 phase and led to increased apoptosis. All these results are consistent with the literature showing that Notch-1 is essential for cell progression beyond the G_1 stage in the cell cycle[40] and that the down-regulation of Notch-1 in pancreatic cancer induced cell cycle arrest in G_0G_1 phase and evoked apoptosis.[18] Even though Notch-1 pathway is likely not the only target of exosomal nanoparticles,[11] it appears that it is an essential component in cell proliferation and apoptosis promoted by exosomal nanoparticles on pancreatic cancer cells.

How presenilin down-regulates the PTEN and GSK-3β activities by phosphorylation is still an open question. Other works support the view that PTEN expression is negatively regulated by Hes-1 in T-acute lymphoblastic leukemia cell lines[41] where mutational loss in PTEN is associated with resistance to γ-secretase inhibitors.[34] In pancreatic cancer, such a loss in PTEN function is not observed. Because the negative control of PTEN expression by Hes-1 as observed in leukemia model is conserved during evolution,[34] it might be that this circuiting either is inefficient or dysfunctions in pancreatic cancer cells. Inhibiting either PTEN or GSK-3β activities rescued cell proliferation. Furthermore, PTEN and GSK-3β seem to be involved in the regulation of Hes-1 expression because inhibiting any of those proteins increases Hes-1 expression. We have shown that upon SOJ-6 cells challenging with exosomal nanoparticles, PTEN and Ser9-phosphorylated GSK-3β form a complex.[11] Because GSK-3β cannot regulate the PTEN phosphorylation on Ser380,[42] it is tempting to speculate that once this complex is formed, Ser9-phosphorylated GSK-3β becomes a substrate for the protein phosphatase activity of PTEN.[43] This is supported by the fact that inhibiting PTEN results in increased phosphorylation of GSK-3β. This also agrees with previous data showing that the PI3K/Akt pathway, albeit activated in the presence of exosomal nanoparticles, is counteracted by the surprising dephosphorylation of GSK-3β.[11] Thus, once activated by dephosphorylation upon cell challenging with exosomal nanoparticles, PTEN in turn can directly activate GSK-3β to repress Hes-1 expression, driving cells towards apoptosis. Finally, consistent with the cell cycle arrest in G_0G_1 phase, a down-regulation of cyclin D1 is observed in cells treated with exosomal nanoparticles,[11] which is in agreement with previous findings demonstrating that the decrease in Hes-1 expression as well as the activation of GSK-3β and PTEN can repress cyclin D1.[43–45] Therefore, it is conceivable that, in cells where the Notch-1 pathway is not the preponderant survival pathway, exosomal nanoparticles could promote other effects such as cell proliferation and/or migration.

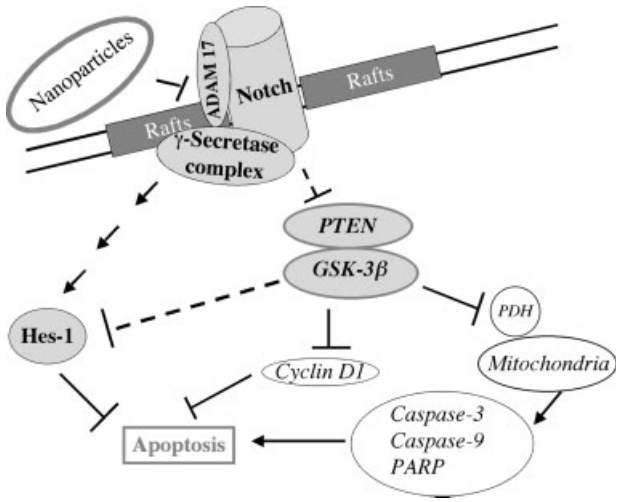


FIGURE 6 – Putative mechanism of action of exosomal nanoparticles on sensitive pancreatic tumoral cells. Exosomal nanoparticles expressed by tumoral cells interact with membrane lipid raft domains of sensitive tumoral target cells to disorganize the Notch-1 receptor complex. This complex implicates ADAM 17 and presenilin of γ-secretase, partners regulating the Notch-1 survival pathway. The disorganization of the Notch-1 complex: 1–activates PTEN and GSK-3β, which in turn inhibit the activity of the mitochondrial pyruvate dehydrogenase (PDH), 2–leads to decreased expressions of Hes-1 and cyclin D1, and 3–drives cells toward the mitochondria-dependent apoptosis. The involvement of italicized partners has been described.[11] Partners in bold are described in the present study. The broken arrow suggests that the effects of presenilin on Hes-1 may not be direct, in our experimental model.

A way to integrate data in a comprehensive scheme (Fig. 6) is to consider that exosomal nanoparticles (which are rich in cholesterol and sphingomyelin, easily incorporated in raft signaling platforms) interact/fuse with lipid raft domains of target cells where Notch-1, ADAM 17, and γ-secretase complex localize. This leads to the inhibition of the Notch-1 pathway, which is particularly sensitive to its lipid microenvironment.[22,23] Presenilin (γ-secretase)

inhibition down-regulates phosphorylation of pro-apoptotic PTEN and GSK-3β leading to their activation. Lastly, GSK-3β inhibits mitochondrial pyruvate dehydrogenase (PDH) on the one hand, and in contrast down-regulates Hes-1 and cyclin D1 expression to drive cells towards apoptosis *via* the mitochondria-dependent pathway.[11]

Notch-1 signaling plays a central role in cell fate during embryonic development of the pancreas where it controls cell differentiation.[28] Elevated expression of Notch-1 in pancreatic cancer leads to the accumulation of undifferentiated precursor cells[46] whereas down-regulation of Notch-1 decreases cyclin D1 and Bcl-2 protein expression to contribute to pancreatic cancer cell apoptosis.[18] Furthermore, aberrant expression of ADAM 17 increases the malignant potential in human pancreatic ductal adenocarcinoma.[47] Exosomal nanoparticles are thought to serve as antigen source and to boost the immune response.[48] However, recent evidence shows that exosomal nanoparticles from tumoral cells may exert a broad array of detrimental effects on the immune system[49] along with the expression of factors involved in angiogenesis promotion and chemoresistance.[50] Altogether these findings support the notion that tumoral exosomal nanoparticles may favor tumor development. We now found that tumoral exosomal nanoparticles are able to discriminate differentiated from undifferentiated tumoral cells. Thus they can help the tumor to eliminate differentiated cells expressing low levels of Notch-1 partners and, as a consequence, indirectly favor the growth of progenitor cell population required for pancreatic cancer development.[17,18]

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