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Pancreatic cancer-derived S-100A8 N-terminal peptide: A diabetes cause?

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Abstract

Background: Our aim was to identify the pancreatic cancer diabetogenic peptide.
Methods: Pancreatic tumor samples from patients with ($n=15$) or without ($n=7$) diabetes were compared with 6 non-neoplastic pancreas samples using SDS-PAGE.
Results: A band measuring approximately 1500 Da was detected in tumors from diabetics, but not in neoplastic samples from non-diabetics or samples from non-neoplastic subjects. Sequence analysis revealed a 14 amino acid peptide (1589.88 Da), corresponding to the N-terminal of the S100A8. At 50 nmol/L and 2 mmol/L, this peptide significantly reduced glucose consumption and lactate production by cultured C_2C_{12} myoblasts. The 14 amino acid peptide caused a lack of myotubular differentiation, the presence of polynucleated cells and caspase-3 activation.
Conclusions: The 14 amino acid peptide from S100A8 impairs the catabolism of glucose by myoblasts in vitro and may cause hyperglycemia in vivo. Its identification in biological fluids might be helpful in diagnosing pancreatic cancer in patients with recent onset diabetes mellitus.

Keywords: Pancreatic cancer; Diabetes mellitus; S100A8; Chronic pancreatitis; Myoblasts; Apoptosis

1. Introduction

While some epidemiological studies indicate that diabetes mellitus, which occurs in about 80% of patients with pancreatic adenocarcinoma [1,2], is a risk factor for this tumor type, others suggest that pancreatic adenocarcinoma causes diabetes mellitus [3,4]. Although diabetes mellitus, especially when insulin treated, may predispose patients to pancreatic cancer, findings in epidemiologic, clinical and experimental studies have demonstrated that this metabolic alteration is caused by pancreatic cancer [5–10]: following tumor excision, glucose tolerance often ameliorates, sometimes returning to normal [2,5]. Moreover, mice treated with pancreatic cancer cell conditioned media develop hyperglycemia [6].

The pathophysiological mechanism underlying pancreatic cancer-associated diabetes probably depends on alterations in beta cell function and glucose metabolism occurring in peripheral tissues. Ding et al. [9] have demonstrated that pancreatic cancer cell conditioned media can dissociate insulin from amylin secretion in beta-cells. Elsewhere, we demonstrated that the C-peptide response to physiological stimuli (e.g., meal or i.v. glucagon) is reduced in pancreatic cancer patients [11]. Furthermore, glycolysis is impaired in hepathocytes cultured in pancreatic cancer cell conditioned media, probably because these media reduce glycolysis, causing a metabolic shift at the triose level sustained by the accumulation of the tryglycerides synthesis intermediate 1,2-diacylglycerol [10].

Pancreatic cancer also causes glucose metabolic alterations in skeletal muscle cells: due to glycogen phosphorylase activation and glycogen synthase inhibition, a skeletal muscle glycogen breakdown prevails over glycogen synthesis, the transcription

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levels of these enzymes being unaffected [12]. We recently demonstrated in vitro that pancreatic cancer cell conditioned media do not alter myoblastic transcription levels of several glycolytic enzymes; nor do they alter the levels of enzymes involved in glycogen synthesis or degradation, although they markedly affect glucose metabolism [13]. The glucose consumption by pancreatic cancer conditioned myoblasts is enhanced, as is lactate production. This effect, also achieved by incubating myoblasts with pancreatic tumor homogenates, is magnified in homogenates from patients with pancreatic cancer-associated diabetes mellitus.

Furthermore, in the skeletal muscle of pancreatic cancer patients, and in pancreatic cancer conditioned myoblasts, proteolysis overcomes protein synthesis, and this probably contributes to cancer cachexia [13,14], suggested to be caused by the proteolysis inducing factor (PIF) peptide, isolated form pancreatic tumors [14,15]. Pancreatic cancer-associated diabetes, in turn, is believed to be caused by an as-yet unidentified low-molecular-weight peptide [9,10,16].

The aim of the present study was therefore to identify and characterize the pancreatic cancer associated diabetogenic factor.

2. Materials and methods

We studied 22 patients (9 males, 13 females; age range 41–83 years) with and 6 patients (4 males, 2 females; age range 28–65 years) without pancreatic cancer, who had chronic pancreatitis (2 cases), benign stenosis of the Wirsung duct (1), duodenal leiomyosarcoma (1), duodenal adenocarcinoma (1) and pancreas divisum (1). Pancreatic tumor stage was stage II (3 cases), stage III (13 cases) and stage IV (6 cases). Diabetes mellitus, diagnosed following the American Diabetes Association criteria [17], was found in 15 patients with pancreatic cancer and in 1 with chronic pancreatitis. Two of the 15 pancreatic cancer patients with diabetes mellitus were insulin treated and three received oral anti-diabetics. Insulin plasma levels were significantly reduced (<2.0 mU/L) in seven (including the 2 insulin-treated pts) and significantly enhanced (>24 mU/L) in one pancreatic cancer patient, but were within the reference range in all the other subjects. All patients underwent abdominal surgery, with intraoperative pancreatic sampling: from pancreatic cancer patients, two tissue samples were obtained, one from the tumor mass and the other from the adjacent disease-free pancreatic tissue; from patients without pancreatic cancer, one pancreatic sample was obtained. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until laboratory analysis.

Neoplastic and non-neoplastic tissue samples were homogenized (1:5 w/v) in ice-cold phosphate-buffered saline (PBS) pH 7.4. In tissue homogenates, the total protein content was measured using Bradford's method (Bio-Rad Laboratories S.r.l., Milano, Italy) [18]. Proteins (100 μg) were denatured in Laemmli's buffer [60 mM Tris–HCl (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 4.6% SDS, and 0.003% bromophenol blue], and separated using the 16.5% discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) system [19]. Electrophoresis was performed at 10 °C (Cryostats Bath, MPM Instruments Srl, Bernareggio, Milano, Italy) for 16 h at 80 V. The protein bands were fixed for 1 h in a solution containing 50% methanol and 10% acetic acid, before being stained with 0.025% Brilliant blue G (Sigma Chemical Co., USA) in 10% acetic acid. De-staining was achieved in 10% acetic acid. The SDS-PAGE separated proteins of a pancreatic tumor sample from a diabetic patient were electro-blotted on a polyvinilydene difluoride (PVDF) membrane (Hybond P, Amersham, Les Ulis, France) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories S.r.l., Milano, Italy). After Coomassie staining of the PVDF membrane, the peptide band to be investigated was cut and loaded into the sample cell of a Procise mod 491 Protein sequencer (Applied Biosystems, Farmington, MA, USA). Automated Edman degradation was carried out for 20 cycles following the manufacturer's instructions. The peptide was then synthesized by Primm s.r.l. (Milano, Italy) at a purity grade of more than 95% (reversed-phase high-performance liquid chromatography).

Three pancreatic cancer cell lines (BxPC3, CAPAN-1 and MIA PaCa 2) and the myogenic mice cell line C_2C_{12} were used. Cells were cultured in DMEM with added 0.1% gentamycin and 10% fetal calf serum (FCS) (BxPC3, MIA PaCa 2 and C_2C_{12}) or RPMI with added 0.1% gentamycin and 20% FCS (CAPAN-1). Harvested cells (500,000), diluted in 50 μL phosphate-buffered saline pH 7.4, were lysed and loaded onto SDS-PAGE gels. For the experiments with synthesized peptide, 60,000 C_2C_{12} cells were seeded in each well of a 24-well culture plate and incubated with low glucose (5.4 mmol/L) DMEM, plus 10% FCS for 24 h.

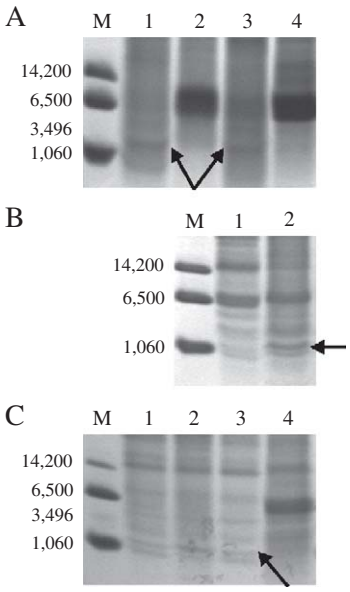

Fig. 1. SDS-PAGE, obtained in cancer tissue samples of patients with or without diabetes mellitus (A), in pancreatic cancer and adjacent disease-free tissue samples from one patient with cancer-associated diabetes (B), in pancreatic cancer cell line lysates or a pancreatic tissue sample from a patient with chronic pancreatitis (C). (A) M = molecular weight marker; 1 and 3 = tumor tissues from patients with diabetes mellitus; 2 and 4 = tumor tissues from patients without diabetes mellitus. (B) M = molecular weight marker; 1 = disease-free tissue sample adjacent to the tumor; 2 = tumor. (C) M = molecular weight marker; 1 = BxPC3; 2 = CAPAN-1; 3 = MIA PaCa 2; 4 = pancreatic tissue sample from one patient with chronic pancreatitis. Arrows indicate the peptide band of 1500 Da, detected in cancer tissues from patients with diabetes and in cancer cell line lysates.

Table 1
Glucose levels (mean±S.D.) measured in control myoblasts and myoblasts incubated with increasing peptide concentrations

	Hours of incubation				MANOVA	
	0	24	48	72	Within subjects	Between subjects
Control (n=13)	25±2.4 mM	23±1.2 mM	17±0.6 mM	13±1.1 mM	F=5.23, p<0.05	F=0.174, p: ns
2 mM Peptide (n=4)	23±3.8 mM	21±2.2 mM	18±0.4 mM	16±0.8 mM		
Control (n=15)	24±3 mM	21±3.2 mM	16±3.6 mM	11±3.9 mM	F=2.4, p: ns	F=1.7, p: ns
1 mM Peptide (n=7)	21±3.6 mM	18±4.8 mM	14±6.3 mM	11±6.9 mM		
Control (n=20)	23±3.4 mM	21±3.1 mM	15±3.2 mM	10±3.4 mM	F=0.13, p: ns	F=2.86, p: ns
500 μM Peptide (n=8)	21±3.2 mM	18±3.6 mM	13±4.4 mM	8±3.9 mM		
Control (n=8)	20±0.7 mM	19±0.8 mM	15±1.2 mM	10±1.2 mM	F=0.1, p: ns	F=0.18, p: ns
300 μM Peptide (n=5)	20±0.9 mM	18±0.5 mM	15±1.4 mM	9±1.6 mM		
Control (n=7)	20±1.2 mM	19±1.2 mM	16±1.6 mM	10±1.1 mM	F=1.76, p: ns	F=0.41, p: ns
150 μM Peptide (n=4)	20±1.8 mM	18±1.5 mM	16±0.7 mM	11±2.3 mM		
Control (n=13)	21±0.8 mM	19±0.9 mM	16±1.8 mM	11±2.2 mM	F=0.98, p: ns	F=1.19, p: ns
75 μM Peptide (n=8)	20±0.4 mM	19±0.8 mM	16±1.6 mM	9±2.9 mM		
Control (n=12)	20±0.6 mM	19±0.9 mM	16±1.7 mM	11±3.1 mM	F=0.62, p: ns	F=0.69, p: ns
35 μM Peptide (n=9)	20±0.4 mM	19±1.0 mM	17±1.8 mM	12±4.5 mM		
Control (n=8)	20±0.7 mM	19±1.2 mM	17±1.7 mM	12±3.4 mM	F=0.07, p: ns	F=0.33, p: ns
15 μM Peptide (n=5)	20±0.4 mM	19±0.7 mM	17±1.6 mM	13±4.7 mM		
Control (n=13)	21±0.8 mM		17±2.3 mM	13±3.9 mM	F=0.105, p: ns	F=0, p: ns
7.5 μM Peptide (n=15)	20±0.9 mM		17±2.3 mM	14±4.1 mM		
Control (n=12)	21±0.3 mM	19±0.9 mM	16±2.7 mM	13±3.9 mM	F=0.06, p: ns	F=0.1, p: ns
1 μM Peptide (n=13)	20±0.3 mM	19±1.1 mM	16±3.3 mM	12±2.3 mM		
Control (n=3)	21±0.6 mM	18±0.4 mM	11±0.4 mM	6±0.5 mM	F=0.3, p: ns	F=0.38, p: ns
500 nM Peptide (n=4)	21±0.8 mM	17±0.8 mM	11±0.3 mM	6±0.6 mM		
Control (n=16)	20±0.8 mM	18±1.8 mM	14±4.2 mM	10±5.2 mM	F=0.17, p: ns	F=0.05, p: ns
100 nM Peptide (n=19)	20±0.9 mM	18±2.1 mM	15±4.5 mM	11±5.9 mM		
Control (n=5)	20±1.4 mM	16±1.8 mM	9±1.9 mM	4±2.2 mM	F=3.45, p<0.05	F=1.17, p: ns
50 nM Peptide (n=5)	20±1.5 mM	18±2.8 mM	13±5.1 mM	8±5.5 mM		
Control (n=13)	21±0.4 mM	19±1.1 mM	16±3.1 mM	12±4.1 mM	F=0.16, p: ns	F=0.44, p: ns
10 nM Peptide (n=14)	21±0.4 mM	19±1.1 mM	17±2.6 mM	13±3.9 mM		
Control (n=3)	20±0.3 mM	19±0.8 mM	16±2.5 mM	14±3.9 mM	F=0.03, p: ns	F=0.04, p: ns
1 nM Peptide (n=5)	20±0.4 mM	19±0.9 mM	16±2.4 mM	14±4.1 mM		

For each concentration, the corresponding control values, obtained in the same experimental runs, are reported. Each experiment was repeated at least three times and the number of data used in each statistical analysis is reported in brackets. No data were available at 24 h of incubation for the 75 μM peptide. The statistical analysis was made using MANOVA.

The media were then replaced with supra-physiological glucose (20 mmol/L) DMEM, plus 10% FCS, and the pure peptide in increasing concentrations (from nmol/L to 2 mmol/L). Media were collected after 24, 48 and 72 h incubation. All experiments were made in triplicate, a set of at least three separate experiments being performed. Control myoblasts were run throughout each experiment. Glucose and lactate were measured within 3 h of collection using a colorimetric method on an automatic analyzer (Dimension RxL, Dade Behring, Milan, Italy).

For cell growth experiments, 600 C_2C_{12} cells/well were seeded in duplicate in a 96-well culture plate and incubated with control medium (DMEM) and different amounts of peptide. Viable cells were estimated after 24, 48 and 72 h using the XTT cell proliferation assay (Roche Diagnostics GmbH, Germany). At least three different sets of experiments were performed.

For apoptosis experiments, 1×10^6 C_2C_{12} myoblasts were plated in Petri dishes (80 cm^2 growth area) and incubated in DMEM (negative control cells), in DMEM added with 0.01 mmol/L of the apoptosis inducer, 5-fluorouracil (5-FU-positive control cells) or 50 nmol/L peptide (tested cells). After 28 h incubation, cells were scraped, washed once with PBS and re-suspended at a concentration of 2×10^6/100 μL in the ice-cold Cell Lysis Buffer provided with the assay kit for caspase-3 activity determination (Calbiochem, Germany). Caspase-3 activity was measured in cell lysates following the manufacturer's instructions. Seven different negative control cells plates, five different tested cells plates and three different positive control cells plates were used to determine caspase-3 activity.

Electrospray ionization (ESI)-mass spectra were acquired using a ion trap LCQ Deca instrument (Thermo Electron, USA). Negative ion ESI-mass spectra of a 10^{-5} mol/L solution (50:50 water/acetonitrile) were obtained by direct infusion using a syringe pump at 8 μL/min. The operative conditions were capillary temperature=230 °C; capillary voltage=−15 V; source voltage=−5 kV.

Peripheral blood mononuclear cells were isolated from the blood of healthy donors by Ficoll-Hypaque gradient centrifugation (Histopaque, Sigma-Aldrich Inc., USA). Monocytes, purified using the MACS CD14 isolation kit (Mylteni Biotec, Bergisch Gladbach, Germany), were cultured in six-well plates ($1–2\times10^6$ cells/mL) in fresh complete medium (RPMI with 10% FCS and 1% glutamine) overnight. Media were then replaced with the following: (1) fresh complete medium (control monocytes); (2) 4 days CAPAN-1 conditioned medium (conditioned monocytes); (3) fresh complete medium with 1 and 5 μg/mL lipopolysaccharide (LPS stimulated control monocytes); (4) 4 days CAPAN-1 conditioned medium with 1 and 5 μg/mL LPS (LPS stimulated conditioned monocytes). Media were collected

Table 2
Lactate levels (mean±S.D.) measured in control myoblasts and in myoblasts incubated with increasing peptide concentrations

	Hours of incubation				MANOVA	
	0	24	48	72	Within subjects	Between subjects
Control (n=13)	3±0.2 mM	7±1.5 mM	16±4.2 mM	26±5.5 mM	F=5.72, p<0.05	F=4.9, p<0.05
2 mM Peptide (n=4)	3±0.1 mM	6±2.5 mM	11±6.4 mM	17±8.4 mM		
Control (n=15)	3±0.2 mM	8±1.8 mM	18±5.2 mM	27±6.4 mM	F=4.38, p<0.05	F=3.14, p: ns
1 mM Peptide (n=7)	3±0.1 mM	6±3.1 mM	14±8.3 mM	19±10.8 mM		
Control (n=20)	3±0.2 mM	7±1.7 mM	17±5.5 mM	26±6.9 mM	F=0.03, p: ns	F=0.03, p: ns
500 μM Peptide (n=8)	3±0.2 mM	7±2.0 mM	16±6.5 mM	25±9.1 mM		
Control (n=8)	3±0.2 mM	6±0.9 mM	11±2.0 mM	20±6.2 mM	F=0.06, p: ns	F=0.04, p: ns
300 μM Peptide (n=5)	3±0.2 mM	6±0.8 mM	11±2.5 mM	21±8.2 mM		
Control (n=7)	3±0.3 mM	5±0.6 mM	9±2. 1 mM	17±0.5 mM	F=2.87, p: ns	F=1.61, p: ns
150 μM Peptide (n=4)	3±0.7 mM	5±1.0 mM	8±2 9 mM	13±6.1 mM		
Control (n=13)	3±0.6 mM	5±0.9 mM	10±2.9 mM	18±5.6 mM	F=1.01, p: ns	F=0.79, p: ns
75 μM Peptide (n=8)	3±0.4 mM	5±1.1 mM	11±2.8 mM	21±5.8 mM		
Control (n=12)	3±0.2 mM	5±0.9 mM	10±3.0 mM	18±6.3 mM	F=0.21, p: ns	F=0.21, p: ns
35 μM Peptide (n=9)	3±0.2 mM	5±1.1 mM	9±2.9 mM	17±7.6 mM		
Control (n=8)	3±0.2 mM	5±0.5 mM	8±2.4 mM	15±3.2 mM	F=0.14, p: ns	F=0.10, p: ns
15 μM Peptide (n=5)	3±0.2 mM	5±0.7 mM	8±3.4 mM	14±4.4 mM		
Control (n=13)	3±0.6 mM		9±4.5 mM	17±9.4 mM	F=0.19, p: ns	F=0.10, p: ns
7.5 μM Peptide (n=15)	3±0.4 mM		9±4.8 mM	16±8.9 mM		
Control (n=12)	3±0.6 mM	6±1.7 mM	11±6.2 mM	20±10.8 mM	F=0.31, p: ns	F=0.28, p: ns
1 μM Peptide (n=13)	3±0.5 mM	6±1.6 mM	12±6.5 mM	22±10.4 mM		
Control (n=3)	3±0.2 mM	10±1.1 mM	25±1.0 mM	35±0.8 mM	F=0.63, p: ns	F=2.24, p: ns
500 nM Peptide (n=4)	3±0.2 mM	9±1.2 mM	24±2.6 mM	34±1.0 mM		
Control (n=16)	3±0.5 mM	7±2.6 mM	14±8.7 mM	24±11.8 mM	F=0.03, p: ns	F=0.002, p: ns
100 nM Peptide (n=19)	3±0.4 mM	7±2.5 mM	14±7.9 mM	24±10.3 mM		
Control (n=5)	3±0.1 mM	10±0.8 mM	26±1.0 mM	36±1.2 mM	F=6.80, p<0.05	F=9.10, p<0.05
50 nM Peptide (n=5)	3±0.1 mM	8±2.3 mM	19±6.0 mM	29±4.5 mM		
Control (n=13)	3±0.6 mM	6±2.3 mM	12±7.5 mM	21±10.9 mM	F=0.23, p: ns	F=0.04, p: ns
10 nM Peptide (n=14)	3±0.4 mM	6±1.8 mM	10±6.2 mM	21±10.9 mM		
Control (n=3)	3±0.5 mM	6±2.6 mM	10±6.5 mM	21±15.3 mM	F=0.003, p: ns	F=0.005, p: ns
1 nM Peptide (n=5)	3±0.3 mM	6±2.5 mM	10±5.7 mM	20±13.1 mM		

For each concentration, the corresponding control values, obtained in the same experimental runs, are reported. Each experiment was repeated at least three times and the number of data used in each statistical analysis is reported in brackets. No data were available for the 75 μM peptide at 24 h incubation. The statistical analysis was made using MANOVA.

after 4 h incubation; the cells were scraped and lysed using a 50 mmol/L Tris–HCl pH 8.5 buffer containing 1 mmol/L DTT, 1 mmol/L EDTA and 10% protease inhibitor cocktail (Sigma-Aldrich Inc., USA). After 30 min centrifugation at 4 °C at 14,000 rpm, the supernatants from cells lysates were collected. Culture media and lysates supernatants were aliquoted and immediately frozen at −80 °C. Total proteins were measured in both culture media and lysate supernatants before SDS-PAGE and Western blotting. For Western blotting, 100 μg proteins were separated onto a 16.5% discontinuous SDS-PAGE and then blotted onto a 0.2-μm PVDF membrane (Bio-Rad Laboratories S.r.l., Milano, Italy). The S100A8 protein was detected using a specific rabbit polyclonal antibody (kindly supplied by Prof. Johannes Roth, Münster, Germany) diluted to 1:300.

The statistical analysis of data was made using repeated-measures analysis of variance (MANOVA), the Fisher's exact test, the Kruskal–Wallis and the Mann–Whitney tests (SPSS statistical software).

3. Results

Fig. 1 shows the findings at SDS-PAGE, obtained in pancreatic cancer tissue samples of patients with or without diabetes mellitus (Panel A), in pancreatic cancer and adjacent disease-free pancreatic tissues samples from patients with pancreatic cancer-associated diabetes (Panel B), in pancreatic cancer cell lines lysates or a pancreatic tissue sample from a patient with chronic pancreatitis (Panel C). The M lines represent the molecular weight marker and the arrows indicate a peptide band of about 1500 Da, which was detected in 7/28 patients, all of whom had pancreatic cancer; 6/7 of these pancreatic cancer patients had diabetes mellitus. On evaluating data from the 17 pancreatic cancer patients not taking drugs for diabetes, a significant association was found between this low-molecular-weight peptide and the presence of diabetes mellitus (Fisher's exact test: $p<0.05$). The 1500-Da peptide was found in cancer specimens, but not in samples from the adjacent disease-free pancreatic tissue obtained from the same patients (Fig. 1, panel B). Furthermore, the 1500-Da peptide was identified in all pancreatic cancer cell line lysates (Fig. 1, panel C).

Proteins from the tumor sample of one patient with pancreatic cancer-associated diabetes mellitus were separated by SDS-PAGE and electro-blotted on a PVDF membrane. The peptide band of about 1500 Da was cut and directly submitted to amino acid sequence by automated Edman's degradation; the sequence obtained was NH_2–MLTELEKALNSIID–COOH, which matched with the N-terminal sequence of the S100A8 protein (http://kr.expasy.org/).

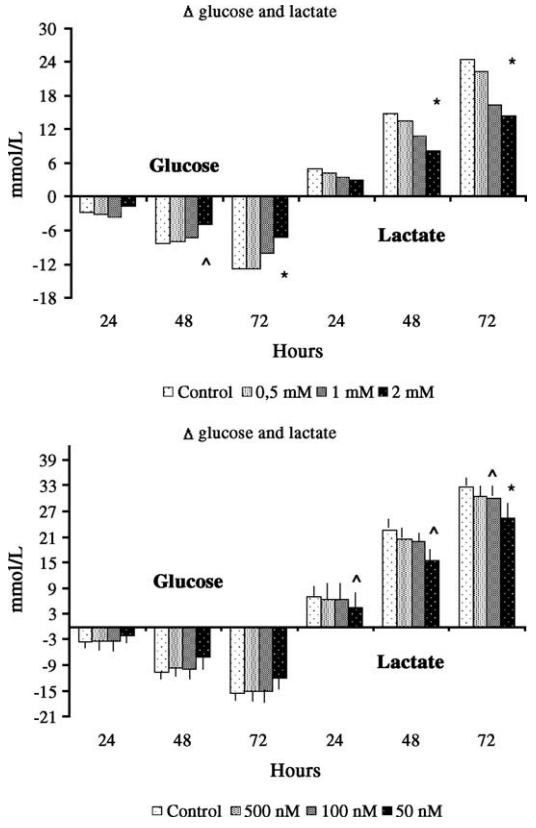

Fig. 2. Glucose and lactate delta (Δ) from basal concentrations found at 24, 48 and 72 h of incubation in the supernatants of myoblasts incubated with high (0.5, 1 and 2 mmol/L) and low (50, 100 and 500 nmol/L) peptide concentrations. *$p<0.01$ with respect to control myoblasts; ^$p<0.05$ with respect to control myoblasts.

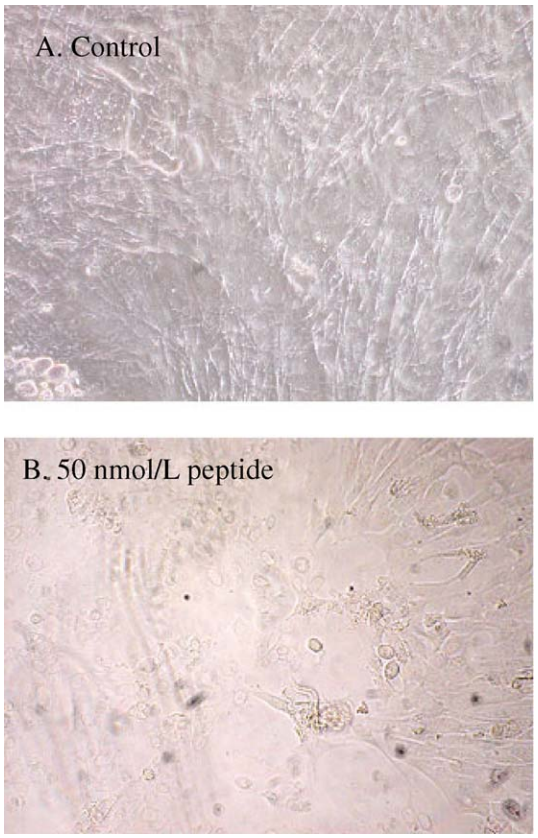

Fig. 3. Morphology of myoblasts after 48 h incubation with control medium (panel A) or a medium plus 50 nmol/L peptide (panel B).

The 1589.88-Da 14 amino acid peptide was then synthesized and its biological effect on myoblastic glucose metabolism tested at increasing concentrations.

Tables 1 and 2 show the glucose and lactate levels found in control myoblasts and in myoblasts incubated with increasing concentrations of the peptide, together with findings made at statistical analysis.

Fig. 2 reports glucose and lactate delta (Δ) from basal concentrations found at 24, 48 and 72 h of incubation in the supernatants of myoblasts incubated with high (0.5, 1 and 2 mmol/L) and low (50, 100 and 500 nmol/L) peptide concentrations. The 2 mmol/L peptide caused a significant decrease in glucose consumption and in lactate accumulation after 48 h (Mann–Whitney $U=8.0, p<0.05$ and $U=4.0, p<0.01$) and 72 h (Mann–Whitney $U=4.0, p<0.01$ and $U=4.0, p<0.01$) incubation. The low-concentrations peptide caused a statistically significant change in the lactate: at a concentration of 50 nmol/L, it inhibited lactate accumulation after 24 h (Mann–Whitney $U=2.0, p<0.05$), 48 h (Mann–Whitney $U=2.0, p<0.05$) and 72 h (Mann–Whitney $U=0$, $p<0.01$); at 100 nmol/L, it caused a significant inhibition in lactate production only after 72 h (Mann–Whitney $U=2.0, p<0.01$).

Myoblastic growth was stimulated only by the 500 nmol/L concentrated peptide, whereas none of the other concentrations affected cell growth (repeated-measures analysis of variance: within-subjects effect: $F=4.48$, $p<0.001$; between-subjects effect: $F=6.51$, $p<0.001$).

Fig. 3 shows the morphology of myoblasts after 48 h incubation in the control medium or in a medium with added 50 nmol/L peptide.

Table 3 reports findings for caspase-3 activity measured in myoblasts incubated in the control medium (negative control) or in a medium with added 0.01 mmol/L 5-FU (positive control) or

Table 3
Measurement of caspase-3 activity in myoblasts incubated for 28 h in DMEM (negative control myoblasts), in DMEM added with 0.01 mmol/L 5-FC (positive control myoblasts) or 50 nmol/L peptide (tested myoblasts)

Caspase-3 activity (pmol/min/μg protein)	Negative control myoblasts	Positive control myoblasts (0.01 mmol/L 5-FU)	Tested myoblasts (50 nmol/L peptide)
Number of repeats	7	3	5
Mean±S.D.	0.198±0.036	0.750±0.115	0.344±0.137
Median	0.203	0.730*	0.252**
Minimum	0.130	0.650	0.240
Maximum	0.250	0.870	0.500

Results, expressed as enzyme activity, represent the mean, the median, and the standard deviation (S.D.), and the minimum and the maximum values obtained from 3 to 7 different experimental sets. The statistical analysis (Kruskal–Wallis test) is also shown.
Kruskal–Wallis test: Chi-square=10.87, $p<0.01$.
* $p<0.05$ with respect to negative control and tested myoblasts.
** $p<0.05$ with respect to negative control myoblasts.

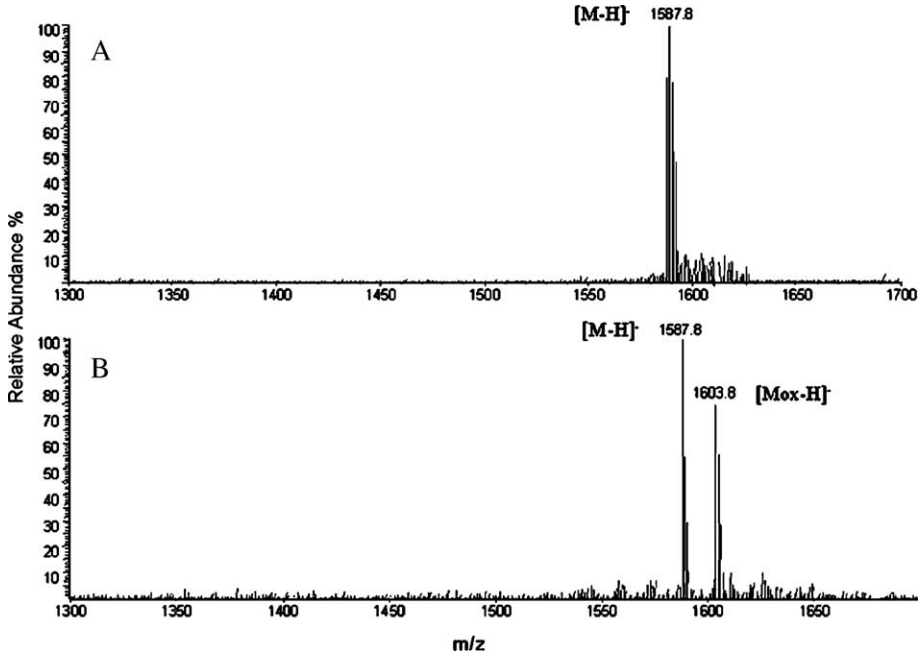

Fig. 4. ESI-mass spectra of active (A) and non-active (B) peptide. Negative ion ESI-mass spectra were obtained from a 10^{-5} mol/L solution.

50 nmol/L peptide. The newly synthesized peptide lost its metabolic and morphologic effects on myoblasts if stored as a powder for more than 1 month at −20 °C instead of −80 °C. We demonstrated, by ESI-mass spectra analysis, that the loss of activity was associated with oxidation. In fact, the mass spectra of the active peptide (Fig. 4, panel A) revealed a peak of 1587.8 Da, while that of the inactive peptide (Fig. 4, panel B) revealed two peaks, one corresponding to the intact molecule and the other, with a molecular mass of 1603.8 Da, showing the difference in mass (16 Da) due to an oxygen molecule.

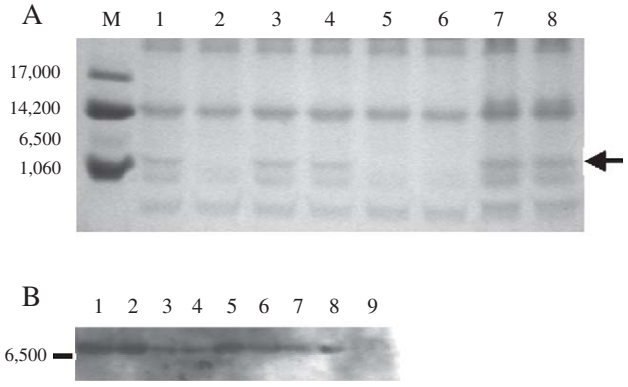

Fig. 5. (A) SDS-PAGE of cell culture media of monocytes incubated with RPMI (control monocytes) or CAPAN-1 conditioned media (conditioned monocytes), stimulated or not stimulated with 1 and 5 μg/mL LPS. M=molecular weight marker; 1=medium from control non-stimulated monocytes; 2=medium from conditioned non-stimulated monocytes; 3 and 4=medium from control LPS 1 and 5 μg/mL stimulated monocytes; 5 and 6=medium from conditioned LPS 1 and 5 μg/mL stimulated monocytes; 7=fresh complete medium (RPMI with 10% FCS and 1% glutamine) after overnight monocyte culture; 8=CAPAN-1 conditioned medium. (B) Western blotting results achieved with the anti-S100A8 polyclonal antibody. 1=CAPAN-1 conditioned monocyte cell lysates; 2=non-conditioned monocytes cell lysates; 3=cell culture medium from CAPAN-1 conditioned monocytes; 4=cell culture medium from non-conditioned monocytes; 5=LPS (1 μg/mL) stimulated CAPAN-1 conditioned monocytes cell lysates; 6=LPS stimulated nonconditioned monocytes cell lysates; 7=cell culture medium from LPS stimulated CAPAN-1 conditioned monocytes; 8=cell culture medium from LPS stimulated non-conditioned monocytes; 9=CAPAN-1 conditioned medium.

Fig. 5 (panel A) shows findings at SDS-PAGE in cell culture media of monocytes incubated with RPMI (control monocytes) or CAPAN-1 conditioned media (conditioned monocytes), stimulated, or not stimulated, with 1 and 5 μg/mL LPS. Fresh, nonconditioned medium and CAPAN-1 conditioned medium not incubated with monocytes were included. The 1500-Da peptide band was shown in the culture media of LPS stimulated and non-stimulated control monocytes and in the CAPAN-1 conditioned medium, but it was no longer detected in the supernatants of CAPAN-1 conditioned monocytes stimulated, or not stimulated, with LPS. Similar results were obtained following SDS-PAGE of monocyte cell lysates. In Fig. 5 (panel B), the results of Western blotting achieved with the anti-S100A8 polyclonal antibody are also shown. This antibody reacted against a protein band of about 6000 Da, corresponding to the entire S100A8 protein, but it did not recognize the 1500-Da peptide, corresponding to the N-terminal sequence of the same protein.

4. Discussion

Glucose metabolic alterations in cultured hepatocytes and myoblasts conditioned by pancreatic cancer cell lines can be reproduced by low-molecular-weight (<10,000 Da) fractions of the same conditioned media [10,13]. Furthermore, findings made at MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) analysis of patients' sera and pancreatic cancer cell conditioned media suggest that a low-molecular-weight peptide is correlated with pancreatic cancer-associated diabetes [16]. To

identify this peptide, we performed SDS-PAGE by focusing on the separation of peptides with a molecular weight of less than 10,000 Da. Pancreatic cancer tissue samples, from patients with or without diabetes mellitus, were analyzed by SDS-PAGE. A peptide band of about 1500 Da, detected in samples from patients with diabetes, was not found in the non-neoplastic pancreas adjacent to the tumors of the same diabetic patients: this result, although obtained from a limited series of patients, too small to draw any definitive conclusion, suggests, however, that the peptide is a tumor-derived product associated with diabetes. This is further borne out by the analysis of (1) a series of pancreatic cancer cell lines, which all presented the peptide following electrophoresis, and (2) pancreatic tissue samples from patients without pancreatic cancer, in none of which was the peptide observed. As these findings were consistent with the hypothesis that the SDS-PAGE-evidenced 1500-Da peptide might be the putative pancreatic cancer-associated diabetogenic factor, sequence analysis was performed on the corresponding SDS-PAGE band using Edman degradation. The resulting amino acid sequence corresponded to a 14 amino acid peptide with a molecular mass of 1589.88 Da, which matched the N-terminal sequence of the S100A8 protein. This protein (also known as Calgranulin A, migration inhibitory factor-related protein (MRP-8), cystic fibrosis antigen (CFAG), calprotectin L1L subunit, urinary stone protein band A, and leukocyte L1 complex light chain), belongs to the family of the S100 calcium binding proteins [20–23]. The major S100 gene cluster, located on chromosome 1q21.3, comprises at least 17 members numbered from S100A1 to S100A17, while the S100B locus is located outside this cluster on chromosome 21q22.3 [23]. Most S100 proteins have a mass of about 9–14 kDa with a conserved C-terminal characterized by a common structural motif, the EF hand having a high affinity for calcium. The N-terminal of these proteins contains a non-canonical EF hand, with two additional amino acids (14 instead of 12) which, in some S100s, binds Ca^{2+} with low affinity [20–23]. This family of proteins plays several biological roles, regulating enzyme activity, cytoskeleton organization dynamics, cell growth and differentiation, and Ca^{2+}homeo stasis. Although S100 proteins are structurally related, differences in their expression profiles and subtle differences in their activity may underlie non-redundant functions. S100A8, in particular, plays a fundamental role in embryogenesis and inflammation [22,24–27]. In cancer cells, S100 proteins seem to have a positive overall effect on cell survival, by modifying the expression of pro-survival genes [28]; altered S100s expression has been found in several types of solid tumor [29–33]. Overexpression of S100s has also been observed in pancreatic cancer [34–38], and S100A8 protein levels were recently found to be significantly increased in tumors compared with normal and pancreatitis tissues [39]. To verify whether the N-terminal peptide of the S100A8 protein has a diabetogenic effect, we tested increasing concentrations of the newly synthesized molecule in cultured myoblasts, known to have a glucose metabolism sensitive to the effects of pancreatic cancer conditioned media [13,40]. Glucose and lactate levels were measured in the supernatants of myoblasts incubated for 24, 48 or 72 h in control medium or in a medium containing different (from nanomolar to millimolar) amounts of the peptide. Both at high (2 mmol/L) and low (50 nmol/L) concentrations, the peptide caused a reduction in glucose utilization and in lactate production over time, suggesting that it interferes with the glucose metabolism of myoblasts. These effects of low and high concentrations on myoblasts glucose metabolism were, at least in part, dose related. The inhibition of glucose utilization at a 2 mmol/L concentration after 48 and 72 h of incubation progressively declined when myoblasts were treated with 1 or 0.5 mmol/L. Accordingly, maximal inhibition of lactate production was recorded after 72 h incubation with 2 mmol/L peptide, the effects of 1 and 0.5 mmol/L peptide being intermediate. Likewise, low peptide concentrations inhibited lactate production in a dose-dependent fashion, the effects being evident after 24 h.

The above findings confirm that the pancreatic cancer-derived peptide can alter glucose metabolism, although in restricted and varying ranges of concentration. The biological effects of different concentrations of the peptide have been reported on by other authors for other S100 proteins, particularly for S100B, which, at nanomolar amounts, enhances neuronal survival and stimulates neurite outgrowth and astrocytic proliferation and at micromolar concentrations causes neuronal and astrocytic apoptosis and stimulates IL-6 secretion through the release of nitric oxide by astrocytes and microglia [21,41].

Like S100B, the S100A8 N-terminal peptide had opposite effects on myoblastic growth at different concentrations. At 500 nmol/L, it enhanced growth, whereas at 2 mmol/L and 50 nmol/L, it caused alterations in the myoblastic morphology: the cells appeared rounded, accumulated at the edge of culture wells and were less differentiated, and some were polynucleated. These morphological alterations were paralleled by an increase in caspase-3 activity, suggesting that the N-terminal peptide of the S100A8 protein triggers the apoptotic cascade. The morphological alterations found by us in treated myoblasts have already been described after exposure to S100B, which causes apoptosis by inhibiting extracellular signal-regulated kinase (ERK) 1/2 [42,43]. The effects of the peptide on myoblastic glycolysis and morphology probably occur independent of calcium binding, since the 14 amino acid peptide studied by us does not contain the EF hand, probably depending on its interaction with cytoskeletal proteins like actin, known to be a target for the interaction of various S100s [21,24,41].

The results obtained in the present study provide further evidence that a small part of a molecule may have significant biological effects, as already demonstrated for S100C/A11 fragments [44]. The effects of the S100A8 N-terminal peptide on glucose metabolism and cell growth were observed when the intact peptide was used, but not when the latter was oxidized, a phenomenon found when the molecule was stored at −20 °C rather than at −80 °C, as demonstrated by ESI-mass spectra analysis. This finding, which demonstrates the specificity of the biological action of the N-terminal of S100A8, is in agreement with previous results obtained with the entire molecule, which fails to exert a chemotactic activity if oxidized by hypochlorite [45].

Although the findings made by us on pancreatic cancer cell lines support the hypothesis that the N-terminal peptide of

S100A8 is produced by tumor cells, we cannot rule out that it derives from inflammatory cells that normally produce the S100A8 protein, monocytes and polymorphonuclear cells, in particular [22]. As demonstrated by Western blot analysis, isolated monocytes release the entire S100A8 in the culture medium. These inflammatory cells were found to release also the N-terminal fragment of this protein. However, when monocytes were conditioned by pancreatic cancer derived media, this peptide was no longer detected. Overall, our results indicate that the N-terminal peptide of the S100A8 protein is produced and released by pancreatic cancer cells and monocytes, and that it is probably not a degradation product from inflammatory cells. Its disappearance in monocyte media after pancreatic cancer cell conditioning may occur consequent to a feed-back regulation, as already described for the entire S100A8 and S100A9 proteins [22].

In conclusion, the findings made in the present study indicate that the 14 amino acid N-terminal peptide of the S100A8, produced by pancreatic tumor cells and monocytes, impairs glucose metabolism, an alteration frequently encountered in pancreatic cancer.

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