PANCREATIC CANCER

Altered glucose metabolism and proteolysis in pancreatic cancer cell conditioned myoblasts: searching for a gene expression pattern with a microarray analysis of 5000 skeletal muscle genes

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Background and aims: We verified whether conditioned media (CM) from pancreatic cancer cell lines (MIA PaCa2, CAPAN-1, PANC-1, BxPC3) alter glucose metabolism and gene expression profiles (microarray experiment with a platform of 5000 skeletal muscle cDNA) in mice myoblasts.

Methods: Myoblasts were incubated with control or pancreatic cancer CM for 24 and 48 hours.

Results: Lactate significantly increased in CM compared with non-conditioned myoblasts. No variations in expression levels of the main genes involved in glycolysis were found in CM myoblasts. Propionyl coenzyme A carboxylase and isocitrate dehydrogenase 3 beta genes, which encode enzymes of the tricarboxylic acid cycle, were overexpressed, while IGF1R and VAMP5 genes were underexpressed in CM myoblasts. PAFAH1B1 and BCL-2 genes (intracellular signal transduction) and the serine protease cathepsin G (proteolysis), were overexpressed in CM myoblasts. Tyrosine accumulation in CM myoblasts suggested that proteolysis overcomes protein synthesis. Sorcin, actin alpha, troponin T1, and filamin A were underexpressed in CM myoblasts.

Conclusions: Our findings demonstrate that pancreatic cancer cell conditioned media enhanced lactate production and induced proteolysis, possibly by altering expression levels of a large number of genes, not only those involved in protein biosynthesis and degradation or glucose metabolism, but also those involved in the tricarboxylic acid cycle and in vesicle traffic.

A clinical history of recently diagnosed type 2 diabetes is often found in patients with pancreatic adenocarcinoma.^{1–3} Epidemiological data suggest that type 2 diabetes is either a risk factor for pancreatic adenocarcinoma^{2 4–6} or a metabolic impairment strictly associated with and consequent to pancreatic adenocarcinoma itself.^{7 8} Although the two hypotheses are not mutually exclusive, the latter is supported by a body of clinical and experimental data^{1 3 9–14}: sudden onset type 2 diabetes is usually found in patients with pancreatic cancer^{2 3 7 8} and tumour excision either ameliorates glucose tolerance or leads to complete remission of diabetes.^{1 9} Furthermore, in vivo and in vitro experimental studies indicate that pancreatic cancer cell products can alter liver and muscle glucose metabolism and can induce peripheral insulin resistance.^{9–11 14 15}

Pancreatic cancer cell conditioned media (CM) reduce liver glycolysis and favour the triglyceride synthesis pathway.¹⁴ Pancreatic tumour extracts from type 2 diabetic patients reduce skeletal muscle glycogen storage,⁹ possibly enhancing glycogen phosphorylase, while reducing glycogen synthase, mRNA, and enzyme activities.¹⁵ Pancreatic cancer has also been reported to cause an altered beta cell response to physiological stimuli¹⁶ and dissociation between insulin and amylin secretion.^{12 13 17}

It has been suggested that rapidly evolving cachexia, another feature of pancreatic cancer, is caused by tumour cell products, particularly the proteolysis inducing factor, which is released into the bloodstream, targeting muscle cells.^{18–21}

The skeletal muscle mass therefore appears to be significantly involved in patients with pancreatic cancer, being the potential target of various tumour products which can

alter glucose metabolism on the one hand and activate protein degradation on the other.

The aims of this study were to verify whether pancreatic cancer cell CM alter glucose metabolism in mice myoblasts and to compare the gene expression profiles of non-conditioned and conditioned myoblasts using a microarray experiment with a platform of 5000 skeletal muscle cDNA.

EXPERIMENTAL PROCEDURES

Cell cultures

We used four human pancreatic cancer cell lines: two (MIA PaCa 2 and CAPAN-1) were obtained from the American Type Culture Collection (ATCC), PANC-1 was a gift from Professor A Scarpa (University of Verona, Italy), and BxPC3 was kindly provided by Dr A Galli (University of Florence, Italy). The colorectal cancer cell line HT29 was obtained by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The myogenic mice cell line C₂C₁₂ was obtained from frozen batches maintained in our laboratories. MIA PaCa 2, PANC-1, and BxPC3 cells were established from human primary pancreatic adenocarcinomas whereas CAPAN-1 cells were established from a liver metastasis from a pancreatic ductal adenocarcinoma. Cells were kept in

Abbreviations: CM, conditioned media; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate buffered saline; NCM, non-conditioned medium; TC, tumour conditioned; MW, molecular weight; RT-PCR, reverse transcription-polymerase chain reaction; ECHS1, enoyl coenzyme A hydratase; IGF2R, insulin-like growth factor 2 receptor; VAMP5, vesicle associated membrane protein 5; FLNA, filamin A; PCCB, propionyl coenzyme A carboxylase; RPS16, ribosomal protein S16

culture (75 cm² flasks) at 37°C in a humid atmosphere, with 5% CO₂ and 95% air. MIA PaCa 2, BxPC3, HT29, and C₂C₁₂ cells were grown in Dulbecco's modified Eagle's medium (DMEM, 0.1% gentamycin and 10% fetal calf serum (FCS); reagents from Life Technologies, Paisley, UK). PANC-1 were grown in RPMI (Life Technologies, Paisley, UK) (0.1% gentamycin and 10% FCS) whereas CAPAN-1 cells were grown in RPMI (0.1% gentamycin and 20% FCS). Pancreatic and colorectal cancer cell line CM were obtained as follows: 400 000 cells were plated in 75 cm² flask and cultured for seven days in low glucose (5.5 mM) DMEM (0.1% gentamycin and 10% FCS). The media were then collected, centrifuged at 1100 *g*, and stored at 4°C for no more than 24 hours, after which the experiments with myoblasts were performed.

Patient tumour samples

Neoplastic tissue samples were obtained at surgery from six patients (two males, four females; aged 50–75 years) with locally advanced pancreatic adenocarcinoma: three had type 2 diabetes (fasting plasma glucose >126 mg/dl). Samples were stored at –80°C for no more than two months. Each tissue sample was homogenised in phosphate buffered saline (PBS 1:20 w/v) and total protein content was measured (Bradford's method; BioRad Laboratories GmbH, Munchen, Germany).

Experiments with mice myoblasts

Sixty thousand myoblasts were plated in each well of a 24 culture plate and cultured for 24 hours. The medium was removed; after washing with PBS, myoblasts were incubated in a non-conditioned medium (NCM = DMEM (0.1% gentamycin, and 10% FCS)), in pancreatic or colorectal cancer cell CM, or in NCM with tumour tissue homogenates (TC = tumour conditioned) reaching a final tumour derived protein concentration of 1 mg/ml. Glucose and lactate concentrations of NCM, CM, and TC were measured and corrected before the experiments in order to obtain the same starting values. The experiments were performed at a glucose concentration of 20 mM. Media were collected after 24, 48 and, in some experiments, 72 hours. All the experiments were done at least in triplicate.

Experiments with mice myoblasts and U-¹³C-glucose

Three separate experiments using mice myoblasts incubated with NCM and pancreatic cancer cell line CM were made using U-¹³C-glucose (Tracer Technologies INC, Somerville, Massachusetts, USA) (2% of native ¹²C-glucose) in order to follow its metabolic fate through ³-¹³C-lactate enrichment. Supernatants were collected after 24, 48, and 72 hours. Samples were immediately frozen and stored at –20°C until ³-¹³C-lactate and ⁵-¹³C-glucose were analysed.

Experiments with mice myoblasts and fractionated CAPAN-1 conditioned medium

A series of three separate experiments were performed with NCM myoblasts and myoblasts incubated with CAPAN-1 CM and with three fractions of the latter, obtained after ultrafiltration with DIAFLO 10 000 molecular weight (MW) and 30 000 MW (Amicon Millipore Corporation, Bedford, Massachusetts, USA). Fractions were: (1) MW <10 000 Da; (2) MW 10 000–30 000 Da; and (3) MW >30 000 Da.

Biochemical analyses

Total glucose and lactate were measured within three hours from collection by means of a colorimetric method on an automatic analyser (Dimension RxL, Dade Behring, Milan, Italy).

Lactate production, derived from glucose, and glucose utilisation (³-¹³C-lactate from U-¹³C-glucose and ⁵-¹³C-glucose)

were analysed as a N-methyl-N-ter-butyldimethylsilyl-trifluoroacetamide derivative (Pierce, Rockford, Illinois, USA) and as pentacetate derivative (Sigma Chemical, St Louis, Missouri, USA) respectively, using a quadruple gas chromatography-mass spectrometry instrument, operating in EI mode with an Agilent 5973 Network after separation in a DB-17 capillary column (J&W, Folsom, California, USA).²² The ratio between tracer (³-¹³C-lactate from U-¹³C-glucose) and tracee (³-¹²C-lactate from natural ¹²C-glucose) mass in the samples was evaluated by isotope ratio measurement (m/z 264/261). The ratio between tracer (U-¹³C-glucose) and tracee (¹²C-glucose) mass in the samples was evaluated by isotope ratio measurement (m/z 247/242). The fragment that represents glucose labelled species resulted with five ¹³C atoms (2-3-4-5-6-¹³C glucose) and is monitored at 247 m/z. The ratio between tracer and tracee mass is evaluated from isotope ratio measurements as:

$$g \times G^* / 1 + G^* \text{ or } L = 1 \times L^* / 1 + L^*$$

where G* is the tracer glucose concentration from exogenous source, g is the tracee glucose concentration from endogenous source, L* is the tracer lactate from glucose, and l is lactate from endogenous source.

Using a high pressure liquid chromatography procedure, tyrosine concentrations were measured in the supernatants of NCM and pancreatic cancer cell line CM obtained from three separate experiments.

Myoblasts experiments for microarray analysis

Mice C₂C₁₂ myoblasts (1.5 × 10⁶) were plated in Petri dishes (= 10 cm) coated with 1% (w/v) type A gelatin from porcine skin (Sigma, Milano, Italy).

A total of 80 Petri dishes were prepared, and myoblasts were cultured for 24 hours. Myoblasts from 20 Petri dishes were scraped and pooled (basal); media were removed from the remaining 60 Petri dishes, myoblasts were washed with PBS, and then incubated with NCM (20 Petri) or CAPAN-1 CM (40 Petri). After 16 hours of incubation, media were collected from 10 NCM and 20 CAPAN-1 CM while myoblasts were scraped and pooled. For the remaining 30 Petri dishes, this procedure was repeated after 26 hours of incubation. Glucose and lactate in the supernatants were measured. Total RNA from myoblasts was purified following the Trizol standard protocol. A small aliquot of RNA (200 ng) was then used for quantification and quality control, using the RNA 6000 LabChip kit and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California, USA). We routinely obtain a mean quantity of 1 µg of total RNA per 10⁵ myoblasts. Total RNA (15 µg) was retrotranscribed and directly labelled in the presence of Cy3 or Cy5 modified dCTP.

Microarray fabrication

The microarrays used for this work (<http://muscle.cribi.unipd.it/microarrayindex.html>) were constructed by arraying polymerase chain reaction (PCR) amplified c-DNAs obtained from our archive of recombinant bacterial clones, on glass slides. This archive consists of 5000 different clones collected after systematic sequencing of skeletal muscle cDNA libraries containing only the 300–500 bp 3' portions of muscle transcripts.²³

Microarray hybridisation

Microarray hybridisation was carried out in a dual slide chamber (HybChamber; GeneMachines, San Carlos, California, USA), humidified with 160 µl of 3 × SSC. Labelled cDNA was dissolved in 35 µl of the hybridisation buffer, denatured at 95°C for three minutes in a thermal cycler, and applied on the microarray slides presaturated by incubation with hybridisation buffer (Northern Max; Ambion, Austin, Texas, USA) for five minutes at 42°C.

Figure 1 Quantitative reverse transcription-polymerase chain reaction results. Agarose gel electrophoresis of enoyl coenzyme A hydratase (ECHS1), TNNT1, insulin-like growth factor 2 receptor (IGF2R), vesicle associated membrane protein 5 (VAMP5), filamin A (FLNA), propionyl coenzyme A carboxylase (PCCB), and ribosomal protein S16 (RPS16). The starting templates were myoblasts incubated with non-conditioned medium (NCM) or CAPAN-1 conditioned medium (CM) for 0, 16, and 26 hours.

Microarrays were covered with a 22×50 mm coverslip and hybridised overnight at 42°C by immersion in a high precision water bath (W28; Grant, Cambridge, UK). Post-hybridisation washing was performed by serial incubations in buffers with decreasing SSC and SDS concentrations, at 42°C. Two replicates of each experiment were obtained using different microarray slides in which the sample and reference RNA was labelled either with Cy3 or Cy5; fluorochromes were crossed in both combinations.

Labelled total RNA targets prepared from NCM and CAPAN-1 CM myoblasts, at different time of growth (hours), were used in competitive hybridisations according to the following scheme:

- (1) 0 hours NCM versus 16 hours CM myoblasts;
- (2) 0 hours NCM versus 26 hours CM myoblasts;
- (3) 16 hours NCM versus 16 hours CM myoblasts;
- (4) 26 hours NCM versus 26 hours CM myoblasts.

Figure 2 Mean (SD) values for glucose concentrations in non-conditioned (NCM) and pancreatic cancer cell line conditioned myoblasts, obtained after 24 and 48 hours of incubation. Repeated measures analysis of variance: time effects, $p < 0.001$; cell lines effects, $p < 0.01$; time × cell lines, $p < 0.01$. Bonferroni's t test: $p < 0.05$ BxPC3 versus NCM.

Figure 3 Mean (SD) values for lactate concentrations in non-conditioned (NCM) and pancreatic cancer cell line conditioned myoblasts, obtained after 24 and 48 hours of incubation. Repeated measures analysis of variance: time effects, $p < 0.001$; cell lines effects, $p < 0.001$; time × cell lines, $p < 0.001$. Bonferroni's t test: $p < 0.05$ CAPAN-1 and PANC-1 versus NCM; $p < 0.001$ MIA PaCa 2 versus NCM.

Validation of the relative gene expression profiling by reverse transcription (RT)-PCR

We used quantitative RT-PCR to validate the results obtained from microarray experiments. Total RNA (2 µg aliquots) from each sample was used to perform three independent cDNA synthesis experiments in a final volume of 20 µl using oligo-dT primer and SuperScript reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy). Gene specific primers were designed using Primer 3 software in order to amplify fragments of approximately 500 bp in length, close to the 3' end of the transcript. cDNA was amplified in 50 µl PCR reactions for a total of 20, 25, 30, and 35 PCR cycles. Each amplicon was electrophoresed on agarose gel and the intensity of the specific bands quantified using a densitometer (GelDoc 2000; BioRad Laboratories, Milano, Italy). Levels of expression were compared with an endogenous control transcript (enoyl CoA hydratase; ECHS1) (fig 1).

Statistical analysis

Array scanning was carried out using a GSI Lumonics LITE dual confocal laser scanner with ScanArray Microarray Analysis Software, while raw scanner images were analysed with QuantArray Analysis Software (GSI Lumonics, Ottawa, Canada). Normalisation of expression levels was performed with a SNOMAD gene expression data analysis tool, a collection of algorithms directed at the normalisation and standardisation of DNA microarray data, available at <http://pevsnerlab.kennedykrieger.org/snomadnput.html>. Before any other statistical analysis, we performed global mean normalisation across microarray surfaces and local mean normalisation across element signal intensity. In single experiments, the mean of the ratio intensity measures of the two replication experiments was calculated and then, after normalisation, $\log_2$ transformation was performed for each expression level. In contrast, expression values of the two replicates of each experiment were considered as two separate values, and each was then converted by logarithmic transformation. Principal component analysis was applied using J-express, a Java tool available at www.molmine.com/index_p.html.

Results were statistically evaluated using one way ANOVA and Bonferroni's test for pairwise comparisons. Repeated measures analysis of variance with interactions was performed using the general linear models procedure. The main effects were time and cell line CM. When the time by cell line interaction was significant ($p < 0.05$), Bonferroni's t tests of difference between means were performed at the global level of probability of 0.05 (SPSS, version 9.0).

Figure 4 Pattern of $[5-^{13}\text{C}]/[^{12}\text{C}]$ glucose (expressed as 247/242 m/z enrichment) measured from 0 to 72 hours after incubation in non-conditioned (NCM) and pancreatic cancer cell line conditioned myoblast supernatants. Values are mean. Standard errors were less than 0.5 mmol/l.

Figure 5 Pattern of $[3-^{13}\text{C}]/[^{12}\text{C}]$ lactate (expressed as 264/261 m/z enrichment) measured from 0 to 72 hours after incubation in non-conditioned (NCM) and pancreatic cancer cell line conditioned myoblast supernatants. Values are mean. Standard errors were less than 0.5 mmol/l.

Detection of differentially expressed genes

Trials of hybridisation with the same RNA labelled with Cy3 and Cy5 on a microarray slide were used as internal quality controls for the detection of a consistent threshold level. According to these experiments, we adopted a threshold level for the logarithmic transformation of the ratio intensity values of 2.5. Then, we considered as differentially expressed only those genes whose replicated spots resulted in expression values below -2.5 or above + 2.5, respectively.

RESULTS

Figure 2 shows mean (SD) values and statistical analysis (repeated measures ANOVA) of glucose concentrations in

Figure 6 Pattern of glucose (histogram) and lactate (lines) concentrations found in myoblast supernatants after incubation with non-conditioned medium (NCM) or the colorectal cancer cell line HT29 conditioned medium. Values are mean (SD).

Figure 7 Pattern of glucose (histogram) and lactate (lines) concentrations found in myoblast supernatants after incubation with non-conditioned medium (NCM) or with media conditioned with pancreatic cancer tumour homogenates obtained from patients with (D) or without (No D) type 2 diabetes. Values are mean (SD). Repeated measures analysis of variance: time effects, $p < 0.01$ for glucose and $p < 0.001$ for lactate; diabetes effects, $p < 0.05$ for glucose and $p < 0.001$ for lactate; time $\times$ diabetes, $p < 0.01$ for glucose and $p < 0.001$ for lactate. Bonferroni's t test: $p < 0.05$ D and No D versus NCM for glucose; $p < 0.05$ D versus NCM for lactate.

NCM and pancreatic cancer CM myoblasts, obtained after 24 and 48 hours of incubation, compared with control myoblasts (time 0). Glucose concentrations declined slightly under all experimental conditions with time and were

Table 1 Mean (SD) values and statistical analysis (repeated measures ANOVA) for lactate concentrations in myoblasts incubated with non-conditioned (NCM) and CAPAN-1 or fractioned CAPAN-1 conditioned media (CM)

	Lactate (mmol/l)		
	Basal	24 hours	48 hours
NCM	3.37 (0.2)	3.56 (0.33)	4.30 (0.52)
CAPAN-1 CM (*)	3.20 (0.05)	3.90 (0.12)	4.96 (0.35)
CAPAN-1 CM >30 000 Da (*)	3.30 (0.09)	4.01 (0.09)	4.91 (0.27)
CAPAN-1 CM 10-30 000 Da	3.33 (0.16)	3.78 (0.25)	4.41 (0.62)
CAPAN-1 CM <10 000 Da	3.33 (0.19)	3.84 (0.24)	4.74 (0.63)

For each condition, eight experiments were done.
Repeated measures analysis of variance: time effects, $p < 0.001$; cell lines effects, $p < 0.05$; time $\times$ cell lines, $p < 0.05$.
Bonferroni's t test: * $p < 0.05$ versus NCM.

Figure 8 Tyrosine levels measured in supernatants of myoblasts after 48 hours of incubation with non-conditioned medium (NCM) or pancreatic cancer cell line conditioned media. ANOVA one way: $F = 4.23$, $p < 0.05$. Bonferroni's test for pairwise comparisons: $p < 0.05$ CAPAN-1 versus NCM.

statistically significant in BxPC3 conditioned versus non-conditioned myoblasts after 48 hours of incubation.

Figure 3 reports mean (SD) values and findings at statistical analysis (repeated measures ANOVA) for lactate concentrations in NCM and pancreatic cancer CM myoblasts, obtained after 24 and 48 hours of incubation compared with control myoblasts (time 0). Lactate concentration increased in both non-conditioned and pancreatic cancer cell line conditioned myoblasts but the magnitude of this increase was higher in conditioned than in NCM myoblasts (statistically significant in CAPAN-1, PANC-1, and MIA PaCa 2).

Figures 4 and 5 show the pattern of $[5-^{13}\text{C}]/[^{12}\text{C}]$ glucose (expressed as 247/242 m/z enrichment) and of $[3-^{13}\text{C}]/[^{12}\text{C}]$ lactate (expressed as 264/261 m/z enrichment) in non-conditioned and pancreatic cancer cell line conditioned myoblasts. The pattern of tracer lactate in conditioned and non-conditioned myoblasts overlapped that of total lactate, indicating that lactate in myoblast supernatants derived from glucose and not from other metabolic substrates.

Table 1 shows lactate concentrations found in myoblast supernatants after incubation with NCM or fractionated CAPAN-1 CM. Lactate significantly increased in supernatants of CAPAN-1 conditioned myoblasts after 24 and, at a higher magnitude, after 48 hours of incubation. Overlapping results were obtained in myoblasts incubated with the two fractions of CAPAN-1 CM with a low ($<10\,000$ Da) and high ($>30\,000$ Da) molecular weight, but not with the fraction with a molecular weight of $10\,000$ – $30\,000$ Da, although statistical significance was reached only for the high molecular weight CAPAN-1 fraction.

Figure 6 shows mean (SD) values for glucose and lactate concentrations in non-conditioned and colorectal cancer cell line HT29 conditioned myoblasts after 24 and 48 hours of incubation compared with control myoblasts (time 0). No significant difference was found for either analytes (repeated measures ANOVA: NS).

Figure 7 illustrates the pattern of supernatant glucose and lactate concentrations of myoblasts incubated with NCM or medium conditioned with pancreatic tumour homogenates from patient with or without type 2 diabetes. Pancreatic tumour homogenates in the medium of myoblasts caused a significant decline in glucose levels, independent of the presence of type 2 diabetes; lactate concentrations increased in all conditioned myoblasts but significantly in CM tumours from type 2 diabetic patients.

Figure 8 illustrates the pattern of tyrosine concentrations in non-conditioned and pancreatic cancer cell line conditioned myoblasts after 48 hours of incubation. No significant

Figure 9 Reverse transcription-polymerase chain reaction results. The housekeeping gene ECHS1 was used as an internal control. Conditioned media (CM) downregulated genes from microarray experiments: TNNT1, IGF2R, VAMP 5, and FLNA. CM overexpressed genes from microarray experiments: PCCB and RPS16. NCM, non-conditioned medium; ECHS1, enoyl coenzyme A hydratase; IGF2R, insulin-like growth factor 2 receptor; VAMP5, vesicle associated membrane protein 5; FLNA, filamin A; PCCB, propionyl coenzyme A carboxylase; RPS16, ribosomal protein S16.

differences were recorded in tyrosine levels at time 0 (ANOVA one way: $F = 0.38$, NS) or after 24 hours of incubation ($F = 1.09$, NS) (data not shown).

To validate our 3'-cDNA array platform, quantitative RT-PCR was undertaken to quantify the level of expression of some muscle transcripts. To this aim, we selected a set of six genes, four of which were underexpressed (TNNT1, IGF2R, VAMP5, FLNA) while two were overexpressed (PCCB and RPS16) in CAPAN-1 conditioned compared with non-conditioned myoblasts, from microarray experiments. The housekeeping gene ECHS1 was used as an internal control. We found a correlation between the results of the array analysis and that of RT-PCR (fig 9).

Expression levels of several enzymes involved in glycolysis were not altered in CAPAN-1 conditioned compared with non-conditioned myoblasts (hexokinase 1 (HK1), glycogen synthase kinase 3 alpha (GSK3A), glycogen phosphorylase, pyruvate kinase (PKM2), enolase 3 (ENO3), aldolase A (ALDOA), GAPDH, phosphofructokinase (PFKM), pyruvate dehydrogenase, isocitrate dehydrogenase 2 (IDH2), glycogenin (GYG), isocitrate dehydrogenase 3 gamma (IDH3G), succinyl CoA synthetase, succinate dehydrogenase, malate dehydrogenase 1 (MDH1), and phosphoglycerate mutase 2).

Table 2 reports overexpressed and table 3 underexpressed genes in CAPAN-1 conditioned versus non-conditioned myoblasts. Only genes with a known biological function are reported. Expression levels at 16 or 26 hours in comparison with baseline control myoblasts gave the following results: eight genes were overexpressed, nine genes were underexpressed. Expression levels found in 16 or 26 hour conditioned myoblasts versus those of 16 and 26 hour control myoblasts were as follows: a total of 14 genes were overexpressed and nine were underexpressed in conditioned myoblasts.

DISCUSSION

It has been suggested that skeletal muscle mass is targeted by tumour derived substances. These can induce glucose intolerance and atrophy, which is responsible, in part, for the marked cachexia and diabetes frequently encountered in pancreatic cancer patients.^{9 15 18–21} We verified the effects of

Table 2 Overexpressed genes with known biological functions in CAPAN-1 conditioned compared with non-conditioned myoblasts

	Muscle cDNA archive ID	Gene names	Biological process	Expression ratio
Overexpressed after 16 h with respect to NCM at time 0	2-001A01	Ribosomal protein S16 (RPS16)	Protein biosynthesis	3.72
	2-007E12	Fibrillarin (FBL)	rRNA processing	2.69
	2-009C09	APOBEC2	rRNA processing	2.50
	2-026B06	BCL-2 associated athanogene	Antiapoptosis, cell surface receptor linked signal transduction	2.57
Overexpressed after 26 h with respect to NCM at time 0	2-031A06	Transcriptional coactivator (ALY)	Protein complex assembly	2.42
	2-039D12	Peroxisome receptor 1 (PXR1)	Protein-peroxisome targeting	2.41
	2-009A02	PFAH1B1	Neurogenesis, cell motility, lipid metabolism, signal transduction	3.34
	BL-001H08	Cathepsin G (CTSG)	Immune response, proteolysis	5.46
Overexpressed after 16 h with respect to NCM after 16 h	2-001A07	Alpha one globin (HBA1)	Oxygen transport	2.84
	2-005G10	Isocitrate dehydrogenase 3 beta (IDH3B)	Isocitrate metabolism, carbohydrate metabolism	2.95
	2-015D05	Ribosomal protein L22 (RPL22)	Protein biosynthesis	3.06
	2-022G02	ARP1 actin-related protein 1 homolog A (ACTR1A)	Vesicle transport	2.55
Overexpressed after 26 h with respect to NCM after 26 h	2-034C09	U4/U6 associated RNA splicing factor (HPRP3P)	mRNA splicing, mRNA processing	3.04
	BL-009A02	Torsin A (DYT1)	Protein folding, heat shock response	2.95
	BL-010F10	NFKB2	Oncogenesis	3.37
	2-010C12	S100 Calcium binding protein A6 (S100A6)	Cell cycle, cell-cell signalling	2.50
	2-018A07	ATP synthase (ATP5B)	Energy pathways	2.47
	2-018C04	Bridging-integrator protein-1 isoform BIN1+12A (BIN1)	Cell cycle control, cell proliferation, non-selective vesicle transport	3.47
	2-018C10	Ribosomal protein S3A (RPS3A)	Protein biosynthesis	2.63
	2-021E09	Ribosomal protein S21 (RPS21)	Protein biosynthesis	3.42
	2-028D10	Propionyl coenzyme A carboxylase	Fatty acid catabolism	2.61
	BL-003G08	MEM-102 glycoprotein	Defence response	3.71

The table lists the transcripts that were found to be upregulated in myoblasts after 16 or 26 hours of incubation with CAPAN-1 conditioned medium in comparison with myoblasts incubated with control medium (NCM).

pancreatic cancer cell conditioned media on glucose metabolism of cultured mice myoblasts and, using a microarray experiment with a platform of 5000 skeletal muscle cDNA, on myoblast gene expression.

Supernatant glucose concentrations slightly declined in conditioned compared with non-conditioned myoblasts, indicating that glucose consumption is higher in conditioned than in non-conditioned myoblasts. The experiments with

Table 3 Underexpressed genes with known biological functions in CAPAN-1 conditioned compared with non-conditioned myoblasts

	Muscle cDNA archive ID	Gene name	Biological process	Expression ratio
Underexpressed both after 16 and 26 h with respect to NCM at time 0	2-001H02	Ribosomal protein S12 (RPS12)	Protein biosynthesis	-2.96/-3.93
Underexpressed after 16 h with respect to NCM at time 0	2-028H06	Actin alpha	Control of heart, muscle contraction	-2.75
	2-029C09	Sorcin (SR1)	Control of heart and muscle development, small molecule transport, intracellular iron storage, action potential regulation, striated muscle contraction regulation	-2.67
Underexpressed after 26 h with respect to NCM at time 0	2-002H11	Thymosin beta 10 (TMSB10)	Spermatid development	-2.88
	2-023F10	Troponin T1	Muscle contraction regulation	-4.33
	2-027F02	IGF2R	Signal transduction, receptor mediated endocytosis	-5.44
	2-029D01	Ribosomal protein L14 (RPL14)	Protein biosynthesis	-2.56
	BL-010C12	PET112L	Protein biosynthesis	-2.73
	BL-010F12	Ribosomal protein L8 (RPL8)	Protein biosynthesis	-3.18
Underexpressed after 16 h with respect to NCM after 16 h	2-010G10	VAMP5	Muscle development, non-selective vesicle transport	-2.53
	2-017G02	Low density lipoprotein related protein 1 (LRP1)	Pathogenesis, lipid metabolism, cell proliferation	-2.67
	2-018F04	Endothelial differentiation related factor 1 (EDF1)	Developmental processes, cell growth and maintenance	-2.85
	2-036G04	Filamin A, alpha (actin binding protein 280) (FLNA)	Neurogenesis, cell motility, cell shape and cell size control, actin cytoskeleton reorganisation, cell surface receptor linked signal transduction	-3.26
Underexpressed after 26 h with respect to NCM after 26 h	2-005F09	NADUFS2	Complex I (NADH to ubiquinone)	-2.66
	BL-003C11	Dual specificity phosphatase 1 (DUSP1)	Oxidative stress response	-4.01

The table lists the transcripts that were found to be downregulated in myoblasts after 16 or 26 hours of incubation with CAPAN-1 conditioned medium in comparison with myoblasts incubated with control medium (NCM).

pancreatic tumour homogenates confirmed and supported this observation. In common with Li and Adrian,²⁴ we found that conditioned myoblasts released higher amounts of lactate in cell culture medium than non-conditioned myoblasts. A very significant increase in lactate production was obtained from myoblasts conditioned with tumour homogenates from diabetic patients, supporting the hypothesis that pancreatic cancer clinically associated with diabetes can cause an increase in lactate production by myoblasts. This finding seems to be peculiar to pancreatic cancer as no effect on glucose or lactate concentrations was found in myoblasts conditioned with a colorectal cancer cell line (HT29).

To confirm that lactate was derived from glucose and not from other metabolic substrates (for example, amino acids), we performed a series of experiments with stable labelled glucose. All lactate derived from glucose, indicating that most of the glucose taken up into the cell was metabolised to lactate rather than undergoing oxidative phosphorylation or being used for glycogen synthesis. The altered glucose metabolism in myoblasts was different from that previously described by us in conditioned hepatocytes where a reduction in lactate production was found.¹⁴ Tumour products may evoke different metabolic alterations strictly linked to the metabolic pattern of the target cell.^{25–26}

The effect on myoblasts was reproduced not only with the low molecular weight fraction of pancreatic cancer cell conditioned media (<10 000 Da)²⁶ but also with the fraction with a high molecular weight (>30 000 Da). Two hypotheses may be proposed: (1) in the low molecular weight fraction a “metabolically active” fragment from a larger molecule may be present; and (2) two different molecules may cooperate in modifying myoblast glucose metabolism.

To screen for which muscle genes expression was altered in conditioned myoblasts, we performed an experiment using microarray with a platform of 5000 skeletal muscle cDNA, which was validated by quantitative RT-PCR. In agreement with our hypothesis that glycolysis is not impaired in conditioned myoblasts, expression levels of many genes involved in this metabolic pathway did not vary. In contrast, we found a large number of over- and underexpressed genes in conditioned versus non-conditioned myoblasts (tables 2, 3). Some of these genes are potentially involved in modifying glucose metabolism: PCCB and isocitrate dehydrogenase 3 beta (IDH3B) genes, which encode enzymes of the tricarboxylic acid cycle, were overexpressed, and IGFIIR and VAMP5 were underexpressed. Propionyl coenzyme A is derived from oxidation of some amino acids (methionine, isoleucine, and valine) and of unpaired fatty acids²⁷ and it is converted into succinyl coenzyme A by PCCB. IDH3B catalyses the oxidative decarboxylation of isocitrate to succinyl coenzyme A via the intermediate alpha-ketoglutarate. We suggest that the mitochondrial tricarboxylic acid cycle could be accelerated, independent of glycolysis, and the production of succinyl coenzyme A enhanced in conditioned versus non-conditioned myoblasts. An accelerated mitochondrial tricarboxylic acid cycle is associated with reduced muscle glycogen synthesis,²⁸ an event already described in diabetic pancreatic cancer patients^{9–15} and it might cause accumulation of ATP and NADH, which are known inhibitors of pyruvate dehydrogenase.²⁷ This event may explain the accumulation of lactate observed in our experimental model.

The biological function of the other genes whose expression was altered was not directly or indirectly correlated with lactate production, although some may be associated with insulin secretion and glucose transport. In this context, IGFIIR and VAMP5 downregulation in conditioned myoblasts is of particular interest as IGFIIR binds IGFII and, with less affinity, also insulin and IGF1.²⁹ Although the effects of IGFIIR downregulation in muscle tissue are not yet known, it

has been reported that glucose increases IGFIIR at the cell surface which promotes insulin exocytosis in insulin secreting cells.^{30–31} Alterations in gene expression observed by us may also occur in other target cells: insulin secreting cells, where decreased IGFIIR may reduce insulin secretion, as already been observed in patients with pancreatic cancer.¹⁶ VAMP5, which belongs to the family of SNARE proteins, may facilitates GLUT4 translocation from the intracellular pool to the plasma membrane.³² In this context, downregulation of VAMP5 might determine reduced GLUT4 membranous expression, followed by reduced glucose transport.

Overexpression, observed by us, of some genes involved in intracellular signal transduction, including PAFAH1B1 and BCL-2, modifying expression of several ribosomal proteins, may lead to alteration in protein biosynthesis.^{33–36} As we found overexpression in the serine protease, cathepsin G, which is involved in connective tissue degradation and caspase activation,³⁷ we examined protein catabolism by measuring tyrosine in supernatants as tyrosine rapidly equilibrates between intracellular pools and the medium and is neither synthesised nor degraded.³⁸ In our experimental model, tyrosine increased in conditioned myoblasts, indicating that proteolysis overcomes protein synthesis, and this may lead to muscle atrophy. Downregulation of sorcin, actin alpha, troponin T1, and filamin A may contribute towards this atrophy.^{39–40}

In conclusion, our findings demonstrate that pancreatic cancer cell conditioned media enhanced lactate production and induced proteolysis, possibly by altering expression levels of a large number of genes—not only those involved in protein biosynthesis and degradation or glucose metabolism but also those involved in the tricarboxylic acid cycle and in vesicle traffic.

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