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SERUM FROM RATS WITH ACUTE PANCREATITIS INDUCES EXPRESSION OF THE PAP mRNA IN THE PANCREATIC ACINAR CELL LINE AR-42J

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SUMMARY : PAP is a pancreatic secretory protein expressed in the pancreas during the acute phase of pancreatitis. We have investigated the effect of the serum from rats with acute pancreatitis (SAP) on the expression of the PAP mRNA in AR-42J cells. PAP mRNA is strongly induced by SAP in a dose-dependent manner. This induction is abolished by preheating the SAP or diminished by treating the cells with cycloheximide. In addition, amylase but not actin mRNA expression was induced by a different SAP factor. We transfected the AR-42J cells with a chimeric gene containing 1.2 kbp 5'-flanking region of the PAP promoter linked to the CAT reporter gene. The CAT activity was significatively increased in the cells, on treating them with SAP. Our results show : first, SAP contains factors responsible for the PAP mRNA expression and secondly, the cis-acting elements are localized within the 1.2 kbp upstream region of the transcription initiation site.

Acute pancreatitis is a pancreatic disease characterized by oedema, inflammation and cellular necrosis. When that episode is not fatal, it is followed by a morphological and functional restitution of the exocrine and endocrine tissues. The acute phase of pancreatitis is characterized by a pattern of changes in the expression of the secretory proteins. Whereas the expression for most pancreatic enzymes decreased, mRNA levels and protein synthesis of the rat "Pancreatitis Associated Protein" (PAP) increased dramatically (reviewed in ref 1). The factor/s

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and the mechanisms mediating this effect are unknown at the moment. However, during the acute phase of pancreatitis several factors are released in plasma and they could be candidates to this effect. To test this hypothesis and as a first step in identifying this/these factor/s and their action mechanisms, we have investigated the effect of the serum from rats with acute pancreatitis on PAP gene expression *in vitro*.

MATERIAL AND METHODS

Cell culture : AR-42J is a rat pancreatic tumor cell line that was derived from an azaserine-induced tumor of the rat exocrine pancreas (2). The cells were routinely cultured at 37°C in a 5% CO_2, 95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (GIBCO), 4 mM L-glutamine, 50 units/ml of penicillin, and 50 µg/ml streptomycin. When cells reached 80-90% confluence, they were dissociated with 0.05% trypsin and 0.02% EDTA in Puck's saline A and replated into 100-mm Petri dishes.

Serums from rats : Experimental pancreatitis was induced on male Sprague Dawley rats weighing 250 gr by intraductal instilation of 200 µl of 2% taurocholate as described by Lankisch (3). Blood was collected twelve hours later, serum was recovered by centrifugation, filtred throgouth a 0.2 µm membrane and stocked at -80°C. Serum from healthy or sham operated (laparotomy) rats was obtained by the same procedure.

Treatment of the AR-42J cells with serum from rats with acute pancreatitis (SAP), helthy (SH) and sham operated (SSO) rats and serum from fetal calf (SFC) : The AR-42J cells (4 X 10^6) were plated on 100-mm culture dishes. When cells attain 50-60% confluence, the medium was removed and remplaced with fresh meduim. After 3 h, SAP, SH or SSO were added to 10% final concentration or with additional 10% SFC (GIBCO). Cells were then incubated for 24 h. In experiments concerning the heated SAP, the serum was pretreated at 55°C or 65°C during 15 min. In a serie of experiments, cycloheximide (20 µg/ml) was added to the medium 30 min before SAP. In the time course experiments, 10% SAP were used for the indicated times. In the dosis response experiments, AR-42J cell were incubated with either SFC as control (2.5, 5, 10 and 20%), SH (0.6, 1.2, 2.5, 5, 10 and 20%) or SAP (0.6, 1.2, 2.5, 5, 10 and 20%) during 24 h.

Preparation and analysis of RNA : Total RNA from cultured cells was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (4). Twenty micrograms were denatured and loaded onto a denaturing 1% agarose gel. Following electrophoresis, the RNA was vacuum blotted onto a nylon filter (Hybond, Amersham) and baked at 80°C for 2h. Filters were prehybridized for 4 hours at 42°C in a buffer containing 50% deionized formamide, 5X SSPE (SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.5), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.5% SDS, and 200 µg/ml denatured herring sperm DNA. Hybridization was conducted at 42°C for 16-20 hours in the same buffer in the presence of the ^{32}P labelled probe. Then the filters were washed four times for 5 min at room temperature in 2X SSC, 0.1% SDS, twice for 15 min at 50°C in 0.1X SSC, 0.1% SDS, and once for 30 min in 0.1X SSC.

Probes : The PAP probe was a 780 bp cDNA insert of the clone 4R (5). The amylase probe was the 1100 bp cDNA insert of clone pCX100 (6). The β actin and 28S ribosomal RNA probes were cDNA inserts of 600 and 900 bp respectively, cloned and sequenced by us (unpublished). The probes were ^{32}P-labelled by random priming (7) using the Ready to go DNA labelling Kit (Pharmacia).

CAT reporter genes constructs, cell transfection and CAT assays : The rat PAP promoter from -1253 to +10 was generated by PCR using the plasmid P/P which contain a 2859 base pairs Pst I-Pst I genomic DNA fragment cloned in the pBluescript KS$^+$, as a template (8). The two oligonucleotides used included 5'-CTGCAGATTTTCCAGTTAGTC-3' and 5'-TGGATGGTTTGTGAGGACAGA-3' as the 5' and 3' primers respectively. The fidelity of PCR was increased by using low dNTP concentrations [9], 100 ng of DNA plasmid as template and only 7 cycles of DNA amplification. Amplification was performed in 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, and 0.01% gelation) containing 125 µM dNTP, 1% DMSO, 25 pmol of each primer, and 2.5 units of Taq polymerase in a final volume of 50 µl. The reaction times were as follows : First cycle, denaturation at 94°C for 2 min, addition of Taq polymerase, annealing at 55°C for 2 min, and extention at 74°C for 2 min ; next 7 cycles, denaturation at 94°C for 10 sec, annealing at 55°C for 2 min, and extention at 74°C for 2 min ; and last cycle, denaturation at 94°C for 10 sec, annealing at 55°C for 2 min, and extention at 74°C for 10 min. The product was kinased and then blunt-end ligated into the Sal I site (filled in with Klenow polymerase) of the promoterlees vector pCATBasic (Promega) to generate the plasmids -1253/+10-CAT and +10/-1253-CAT. One day after seeding AR-42J cells at a density of 5 X 10^6 cells per 100-mm Petri dish, a calcium phosphate coprecipitate (10) containing 20 µg of plasmid DNA was added to the cells. Twelve hours after the addition of the DNA, cells were subjected to 20% (vol/vol) glycerol for 2 min, and the cells were washed with serum-free medium and transferred to serum-containing medium. Forty-five hours later 10% SH, SAP or SFC was added and incubated for an additional 12 h. At 69 h post transfection cell extracts were prepared using the reporter lysis buffer (Promega). CAT activity was determined using a phase extraction procedure (11).

RESULTS AND DISCUSSION

PAP mRNA was expressed at very low levels in the normal pancreas of the rat. However, PAP mRNA is strongly induced during the acute phase of pancreatitis (5). As in the healthy pancreas, PAP mRNA was only detected in AR-42J cells by RT-PCR (8), suggesting a very low level of expression of this transcript in uninduced conditions. In this work we have used the AR-42J cell line as a model to test the effect of serum from rats with acute pancreatitis (SAP) on the PAP mRNA expression.

Effects of serum from rats with acute pancreatitis on PAP mRNA levels : Figure 1 shows that the expression of PAP mRNA is induced in AR-42J cells after 24 h of treatment with 10% SAP. However, when cells were treated simultaneously with cycloheximide (20 µg/ml) and SAP, this effect was diminished. On the other hand, when the SAP was heated during 15 min at 55°C or at 65°C this induction strongly decreased or disappeared respectively. These results suggest that expression of the PAP mRNA is dependent, at least in part, on the protein neosynthesis and it is mediated by a thermolabil factor/s. Serum from sham operated (SSO) or healthy (SH) rats, but not serum from fetal calf (SFC), induces a poor expression of the PAP mRNA (only observed after longer exposition of the autoradiograms). We also studied the amylase mRNA expression under the same experimental conditions. Interestingly, SAP also induced amylase mRNA expression. When the SAP was heated during 15 min at 55°C or 65°C, the effect remained unchanged. Finally,

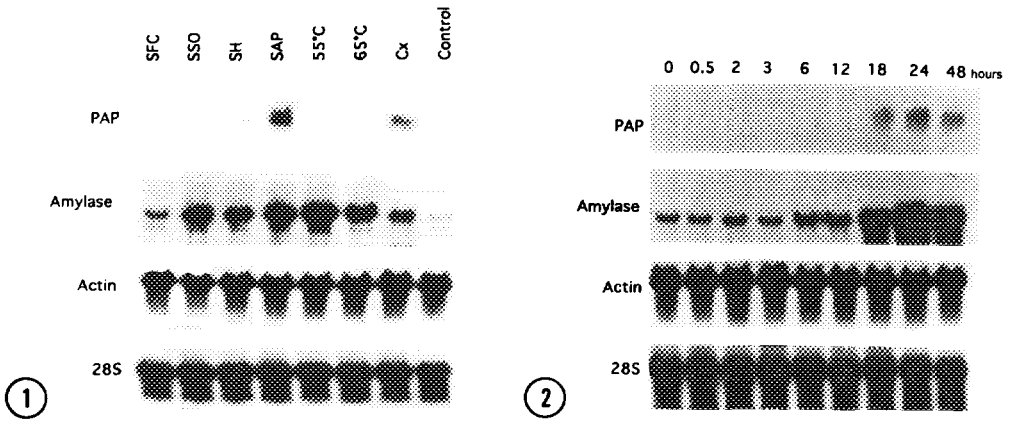


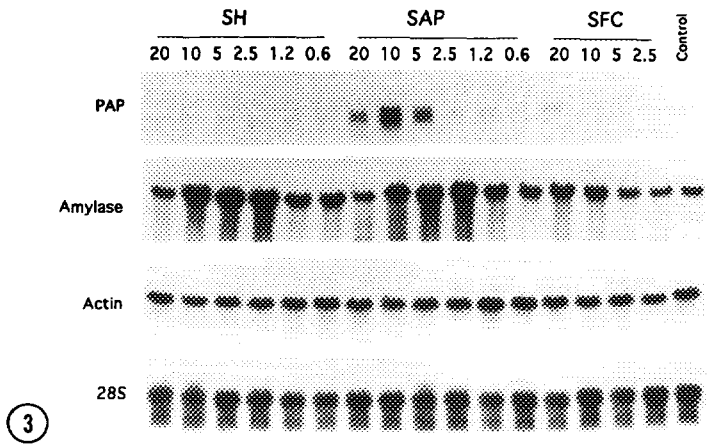
Fig. 1. The effect of serum from fetal calf (SFC), sham operated (SSO) rats, helthy (SH) rats and rats with acute pancreatitis (SAP) on AR-42J cells. 50-60% confluence AR-42J cells were treated for 24 h with 10% of SFC, SSO, SH, SAP or pretreated SAP at 55°C or 65°C during 15 min. Cycloheximide (Cx) was added to the medium (20 µg/ml) 30 min before SAP or cells were untreated (Control). RNA preparation and Northern blot analysis were performed as described under "Material and Methods".

Fig. 2. Time course analysis of the effect of serum from rats with acute pancreatitis (SAP) on AR-42J cells. 50-60% confluence AR-42J cells were treated for the indicated times with 10% SAP. RNA preparation and Northern blot analysis were performed as described under "Material and Methods".

when the cells were treated with cycloheximide and SAP simultaneously, the amylase mRNA induction was decreased. Therefore, amylase mRNA induction is also dependent on the protein neosynthesis but the effect is mediated by a thermoresistent factor/s. This observation strongly suggest that SAP factor/s responsible of the effect for PAP gene expression is/are different from this/these implicated in the amylase gene expression. Serum from sham operated (SSO) or healthy (SH) rats, but not serum from fetal calf (SFC), also induces expression of the amylase mRNA. Actin mRNA expression was unmodified by treatment with SAP, SSO, SH or SFC under our experimental conditions. The 28S ribosomal RNA was measured as control of the comparable amounts in each lane (Figure 1).

Figure 2 illustrates time course experiments in which AR-42J cells were treated for different times with SAP (10%). The SAP-mediated induction of PAP and amylase showed a similar pattern, accumulation of both mRNAs becoming evident between 12 and 18 h of treatment and a plateau being reached after 18 h. Actin mRNA expression was not modified.

AR-42J cells were treated with various concentrations of SAP, SH or SFC to determine the effect on induction of PAP, amylase and actin mRNAs. Figure 3 shows the treatments of cells with 0.6 to 20% SAP or SH by 24 h. PAP mRNA is induced from 5 to 20% SAP, with a maximal expression at 10%. Any effect was obtained with SH or SFC. Amylase mRNA was induced with 2.5 to 10% SAP, however,



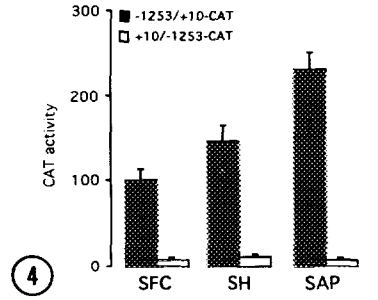


Fig. 3. The effect of different concentrations of serum from helthy (SH) rats, rats with acute pancreatitis (SAP) or fetal calf (SFC) on AR-42J cells. 50-60% confluence AR-42J cells were treated for 24 h with SH (0.6, 1.2, 2.5, 5, 10 and 20%), SAP (0.6, 1.2, 2.5, 5, 10 and 20%) or SFC (2.5, 5, 10 and 20%). RNA preparation and Northern blot analysis were performed as described under "Material and Methods".

Fig. 4. Transient expression of PAP promoter-CAT constructs. AR-42J cells were transfected with 20 µg of -1253/+10-CAT or +10/-1253-CAT plasmids. Transfected cells were treated with SFC, SH or SAP (10%) as described in "Material and Methods". Results were normalized to the activity of the SFC-treated cells to which a value of 100 was assigned. The results presented are the average from at least three independent experiments performed in triplicate.

any effect was observed with 20% SAP. SH induces expression of amylase mRNA when the cells were treated with 2.5 to 10%. No effect was detectable on actin mRNA levels after SAP, SH or SFC treatment.

Effects of serum from rats with acute pancreatitis on upstream regulatory sequences of PAP gene : The first part of this work shows that SAP increase PAP mRNA concentration in AR-42J cells (Figs 1-3). To assess whether or not the 1.2 kbp 5'-flanking region contains cis-acting promoter elements necessary for the induced expression of the PAP gene in AR-42J cells, we constructed a chimeric gene containing this region linked to the coding region of the bacterial chloramphenicol acetyltransferase gene, subsequentely referred to as -1253/+10-CAT. Plasmid +10/-1253-CAT (in reverse orientation) was used as a control. Figure 4 shows the comparison of CAT activity in extracts from AR-42J transfected with the above constructions and incubated with 10% SAP, SH or SFC. Treatments with SAP increase the CAT activity by 2.2 times, whereas with SH only 1.4 times, respect of the SFC-treated cells. These exciting results suggests that a SAP-response element exist within the studied PAP promoter region.

In conclusion, a factor/s that induces PAP mRNA expression in AR-42J cells is present in SAP and, this induction is in part mediated by a neosythesized protein and by a thermolabil factor. In addition, this induction did not result in an

increase of the levels of all mRNAs due to a general effect since actin mRNA levels did not change by SAP, SH or SFC treatment. The factor/s that induces amylase mRNA expression is different from that because this is thermoresistent and is also present in SH. Finally, the cis-acting element responsible of the PAP gene induction by SAP is localized within the 1.2 kbp upstream region of the transcription initiation site. This work establishes a powerfull model for the study of seric factors as well as the cis acting elements and nuclear factors involved in the expression of the PAP gene. We work actually on this way.

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