Arch. Biol. Sci., Belgrade, 61 (1), 9-15, 2009 DOI:10.2298/ABS0901009D

APOPTOSIS OF HUMAN FETAL PANCREATIC ISLETS DURING SHORT-TERM CULTURE

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Abstract — We investigated the influence of short-term culture in vitro on the appearance of apoptosis of human fetal pancreatic islets (HFIs) and its effect on the mass and insulin-secretory capacity (ISC) of β-cells. It was found that apoptosis was present from the end of the culture period, increasing as a function of time and leading to decrease of β-cell mass. At the same time, ISC decreased. The decrease of β-cell mass and ISC may influence significantly the clinical outcome of HFIs transplantation in type 1 diabetic patients.

Key words: Human fetal pancreatic islets, short term culture, apoptosis, insulin-secretory capacity, clinical outcome

UDC 577.24:57.085.577.175.72:616.379-008.64

INTRODUCTION

Today, there is no doubt that transplantation (Tx) of HAIs may produce insulin independence in insulin-dependent type 1 diabetics. The best results (approximately 60%) until now have been achieved by the Edmonton team (Ryan et al., 2001). They used 630 000 HAIs per one person for the alloTx. These results were confirmed in some very experienced centers (Tanioka et al., 1997; Moberg et al., 2002; Ozmen et al., 2002; Shapiro et al., 2003), but not elsewhere (Ault, 2003). Optimal islet graft depends to a great extent on the processes of islet retrieval, preservation, isolation, and purification. For alloTx of HFIs, there are considerably fewer data. Insulin independence has not yet been achieved, but clinically significant secretion of insulin from grafted islets (approximately 150 000±34 000) has been shown, together with improvement of glycoregulation and decrease of the insulin daily dose by up to 60% (partial clinical success) (Djordjević, 1995). These effects were time-limited. The yield of islets and their functional capability depend to a great extent on many factors, including the type of collagenase enzyme, length of incubation with the enzyme, duration of culture, methods used for isolation and purification, etc. The influence of apoptosis of HAIs after Tx has also been cited (Paraskevas et al., 2000). It is generally known that not only during the post-transplant period, but in the pretransplant period as well, apoptosis of HAIs occurs. Apoptotic insults include the influence of inflammatory cytokines (Dealaney et al., 1997; Lakey et al., 2001), metabolic stress (decreased ATP/ADP ratio; activation of AMP kinase), and hypoxia. It is interesting that activation of AMP kinase may trigger not only apoptosis of β-cells in HAIs (Kefas et al., 2003, 2003a), but also inhibition of insulin release (Leclerc et al., 2004). This last finding is of possible interest for our attempt to investigate the eventual connection between apoptosis and ISC of HFIs.

Taking into consideration the aforementioned facts about HAIs and lacking data on HFIs, we set out to estimate the presence of apoptosis and the value of ISC in conditions of short-term culture.

METHODS

Human fetal islets were isolated from fetal pancreata (N=7) with gestational age of 16-24 weeks. Fetal pancreatic tissue weight was 0.476±0.216 g (mean ± SD). Warm and cold ischemia lasted for 52.60±6.75 and 54.0±13.29 min, respectively. Warm ischemia lasted from the beginning of the isolation process till the moment of placing cells in plastic culture flasks,

which were immediately transferred to an incubator. Pancreata were obtained after spontaneous or prostaglandin-induced abortion or after operation (section parva) due to medical reasons. Ethical committee approval and parental consent were obtained.

Islets were isolated under aseptic conditions by a non-automated method using Collagenase IX, 5 mg/ml (SIGMA-ALDRICH) (Jovanović-Peterson et al., 1988; Otonkoski et al., 1991). After semidigestion for 30 min at 37°C, the supernatant was decanted and cells were washed in HBSS (Aplichem-GmbH) solution containing 20 mM Hepes, 5.6 mM glucose, and 0.2% FCS, pH 7.4. The islets were then resuspended in culture medium RPMI 1640 (SIGMA-ALDRICH) containing $Ca(NO_3)_2$ x 4 H_2O 0.1 g/l, $MgSO_4$ (anhydride) 0.0484 g/l, KCl 0.4 g/l, $NaHCO_3$ 2 g/l, NaCl 6 g/l, Na_2HPO_4 (anhydride) 0.8 g/l, glutamine 0.3 g/l, D-glucose 1 g/l, 25 mM HEPES, 10% FCS (fetal calf serum), 100 U/ml penicillin, and 100 μg/ml streptomycin; transferred to plastic culture flasks (Falcon 3013, volume of 50 cm^3); and incubated at 37°C in a 5% CO_2, 95% humid atmosphere for 24 and 48 h.

Approximately 6,000 islets were isolated from one fetal pancreas (the volume of one culture was 10 ml), i.e., the number of islets was approximately 600 islets per 1 ml. One milliliter of the culture was used for determination of apoptosis (mean ± SD = 610±67.54) and the remainder of 9 ml was used for stimulation with high and low glucose concentration. By this approach, determinations of apoptosis and functional capacity of pancreatic islets were performed at the same time at 0-, 24-, and 48-h intervals. The number of islets and viability were determined by staining with dithizone (Akira et al., 2002). Apoptosis was established by TUNEL staining according instructions of the manufacturer (Roche Applied Science in Situ Cell Death Detection Kit Ap Cat No. 11684809910) using the TUNEL staining method of R. Sgonc et al. (1994). The main advantage of this simple and rapid procedure is the use of Fluorescein-dUTP to label DNA strand breaks. This permits detection of DNA fragmentation by fluorescence microscopy directly after the TUNEL reaction prior to addition of the secondary anti-fluorescein-AP conjugate.

The functional capacity of isolated islets was established by static glucose stimulation assay. Islets were incubated for one hour at low and high glucose concentration (1.67 mM and 16.7 mM, respectively) (Mandel, 1998) in HEPES – buffered Hank's balanced salt solution on the first and second day of cultivation. The supernatant of each sample was collected and stored at -18°C. Insulin content was measured by radioimmunoassay (RIA INSULIN PEG), INEP, Belgrade, Serbia. Sensitivity of the assay was 0.60 mU/l, the detection range 0.6-300 mU/l. Insulin release (insulin secretory capacity – ISC) in response to the glucose challenge under static incubation was calculated as the ratio of insulin released during one hour of incubation at high glucose concentration to insulin released during one hour of incubation at low glucose concentration. First of all, the functional secretion rate (FSR) was calculated using the following equation: $FSR = F_1/F_2$, $F_1 = 1.6/(1.6+B+16.6)$ and $F_2 = 16/(1.6+16)$ where: 1.6 = insulin secretion (μU/ml) after one hour of incubation of culture at low glucose concentration (1.6 mM), 16.6 = insulin secretion (μU/ml) after one hour of incubation of culture at high glucose concentration (16.6 mM), and B = basal insulin secretion after one hour of incubation in culture medium without glucose. Secondly, ISC (%) was calculated using the following equation: FSR x 100. The results are given in a table containing basic information on all seven samples of tissue for the control, 24-h, and 48-h groups. A separate table gives the values of ISC and apoptosis for the control, 24-h, and 48-h groups.

As controls, we used the samples taken immediately after the end of the culture procedure (0 h).

Apoptosis was established at time 0, i.e., immediately after the end of the process of isolation of pancreatic islets and introducing them to culture. This time point was marked as the control.

For statistical analysis of differences between the groups, the t-paired test was used.

RESULTS

TUNEL staining of HFIs showed a positive reaction. The mean value (mean ± SD) of apoptosis after 24

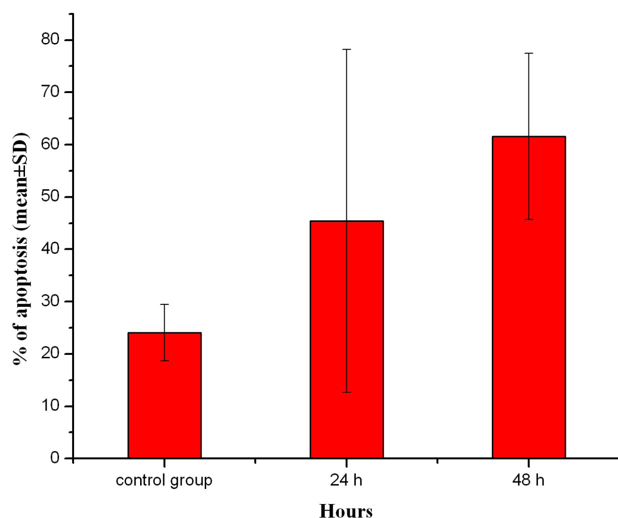

Fig. 1. Mean value ± SD of HFI apoptosis in the control group after 24 and 48h.

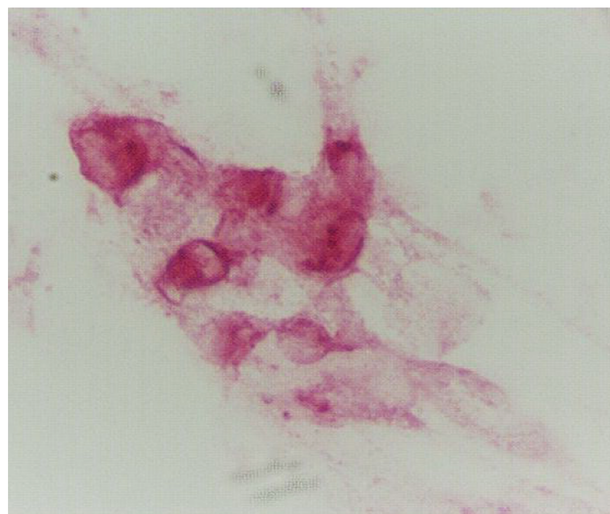

Fig. 2. TUNEL staining of HFIs showing positive reaction after 48 hours. In culture, magnification 800x.

h was 45.44±32.8% and increased to 61.57±15.88% after 48 h, while the value in the control group was 24.04±5.39% (Fig. 1). The increase of apoptosis after 48 h had very high statistical significance in comparison with the control group ($p<0.001$).

The positive TUNEL reaction after 48 h in culture is given in Fig. 2.

The value of ISC (mean±SD) was 405.20±33.64% in the control group, decreased after 24 h to 381.0±33.4%, and remained unchanged at 386.22±36.94% after 48 h (Fig. 3). In comparison with the control group, the decrease after 48 h was statistically significant ($p<0.05$).

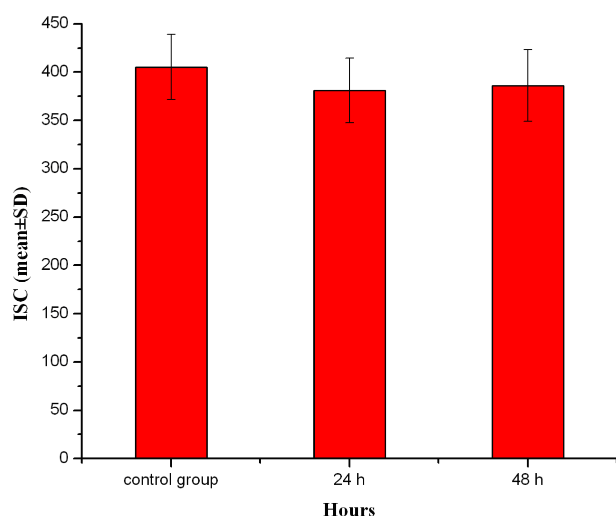

Fig. 3. Mean value ± SD of HFI ISC in the culture in control group after 24 and 48h.

The individual data necessary for estimation of ISC are given in Table 1.

The individual data on apoptosis and FSR values in the investigated period of time are given in Table 2.

DISCUSSION

Even under conditions when a sufficient number of purified HAIs are applied, it is possible to have a Tx that is unsuccessful and unable to achieve insulin independence. Among many other factors, apoptosis is believed to have considerable influence on the positive clinical outcome of Tx of HAIs. It was mentioned earlier that apoptosis also occurs in the pretransplant period, i.e., during the culture of HAIs (Dealaney et al., 1997; Lakey et al., 2000; Kefas et al., 2003, 2003a). It has been shown that interleukin-1b is able to inhibit β-cell function, as well as activate Fas-mediated apoptosis in a nitric oxide-dependent manner, while the insulin-like growth factor (IGF-1) blocks these actions *in vitro* (Giannoukakis et al., 2000). Human fetal pancreas contains IGF-1, which was secreted in a glucose-dependent manner *in vitro* (Hill et al., 1998). It is very interesting

Table 1.

	Insulin secretion (μU/ml/h)						
	culture no.	1.67	B	16.7	F1	F2	FSR
control group	1	81.9	89.1	269.1	0.186	0.751	4.036
	2	65.9	60.4	181.9	0.213	0.75	3.51
	3	41.3	54.7	158.9	0.162	0.743	4.591
	4	47.8	49.1	149.6	0.193	0.752	3.882
	5	61.8	69.8	217.7	0.176	0.757	4.279
	6	52.9	54.3	168.9	0.191	0.756	3.949
	7	49.5	51.9	165.9	0.185	0.761	4.113
24 h	1	69.5	63.8	191.4	0.214	0.75	3.503
	2	29.3	21.5	87.8	0.211	0.803	3.799
	3	84.2	79.5	252.7	0.202	0.76	3.761
	4	65.9	60.4	181.9	0.213	0.75	3.51
	5	63.1	59.3	177.9	0.21	0.75	3.569
	6	74.9	86.2	258.6	0.178	0.75	4.202
	7	71.5	85.3	256.1	0.173	0.75	4.331
48 h	1	54.9	59.1	17.3	0.187	0.752	4.018
	2	24.5	30.6	58.5	0.215	0.656	3.044
	3	74.1	76.5	230.5	0.194	0.75	3.861
	4	52.9	54.3	168.9	0.191	0.756	3.949
	5	49.5	51.9	165.9	0.185	0.761	4.113
	6	71.6	78.3	236.8	0.185	0.751	4.058
	7	72.3	77.5	234.2	0.188	0.751	3.99

Table 2.

	control group			24 h			48 h		
culture no.	APO	FSR	ISC (FSR x 100)	APO	FSR	ISC (FSR x 100)	APO	FSR	ISC (FSR x 100)
1	15.2	4.0369	403.69	8.7	3.503	350.39	46	4.018	401.8
2	20.2	3.51	351.09	80	3.799	379.98	80	3.044	304.43
3	34.1	4.591	459.14	10.7	3.761	376.18	48	3.861	386.14
4	22.7	3.882	388.26	78	3.51	351.09	78	3.949	394.95
5	26.3	4.279	427.98	15.7	3.569	356.93	43	4.113	411.32
6	25.5	3.949	394.95	72	4.202	420.26	73	4.058	405.87
7	24.4	4.113	411.32	53	4.331	433.19	63	3.99	399.06
mean± SD	24.04±5.39		405.204±33.64	45.44±32.8		381.14±33.42	61.57±15.88		386.22±36.94
mean±SE	24.04±1.91		405.204±12.71	45.44±12.4		381.14±12.63	61.57±6		386.22±13.96

that highly purified HAIs are still immunogenic via the mechanism of indirect presentation of antigen (Stock et al., 1991) and able to trigger the apoptosis of β-cells in culture (Frisch et al., 1994). In normal (*in vivo*) conditions, approximately 0.5% of adult β-cells undergo apoptosis. This process is balanced by β-cell replication and, to a lesser content, by β-cell neogenesis (Bonner-Weir, 2000; Lingohr et al., 2002). However, under *in vitro* conditions, apoptosis of HAIs was estimated at up to 50%, together with other noninflammatory pathways. Thus, in one group of experiments, the authors established that normal and diabetic islets had 10.3±3 and 19±5% apoptosis, respectively when culture duration was 3-4 days (Marchetti et al., 2004). It has also been shown that under *in vivo* conditions, the islets from type 2 diabetic donors, in addition to impaired insulin secretion, exhibit increased apoptosis, which causes decreased β-cell mass (Butler et al., 2003). These alterations were associated with increased oxidative stress. Incubation with metformin for 24 h was linked with improved glucose-induced insulin release and reduced apoptosis due to reduction or normalization of several markers of oxidative stress (Marchetti et al., 2004).

In this investigation, we found that the percentage of apoptosis of HFIs was high after 24 h of culture (45.44±32.8%) and even increased to 61.57±15.88% (mean ± SE) after 48 h. We found fewer similar data in the literature for HFIs, especially under *in vitro* conditions. Concerning the presence of apoptosis as high as 24.04±5.39% in the control group of our experiments, the reasons for this may be different. First of all, the process of isolation of damaged cells, but the process of apoptosis occurred at the same time. Secondly, fetal pancreata contain a mechanism of apoptosis avoidance that leads to active proliferation. In this proliferation, Bcl-XL and Ncl-1 proteins may play an important role. Thirdly, the presence of Bax, a proapoptotic protein, plays the opposite role (Kobayashi et al., 2000). It is possible that the causes may be the same as for HAIs. It is generally known that basal NO production by islets, as well as detectable caspase-3 activity, may be a natural response to trauma of islets during pancreas procurement and/or during the islet isolation procedure. On the other hand, the removal of islets from the pancreas may deprive them of a trophic factor that suppresses proapoptotic events, i.e., nitric oxide formation. Clarification of this question will be the aim of our next investigations. Like most authors, we used the TUNEL staining method to establish the presence of apoptosis. A novel method for specific identification of adult β-cell apoptosis was proposed using laser scanning cytometry (LSC) at the mitochondrial level (Ichii et al., 2005). This method may also prove to be useful for our future investigation with HFIs in short-term and long-term culture. It is also necessary to investigate the possibilities preventing of apoptosis of HFIs, as shown for HAIs using the two layer method for pancreas preservation (which leads to increase of ATP content) or gene therapy, including over-expression of BCL-2 (Contreras et al., 2002). Finally, if we achieve successful protection of β-cells from apoptosis, we may even expect increases in the ISC of HFIs, which was decreased in comparison with the control group in the presence of a high percentage of apoptosis in our investigation.

The possible reasons for our findings are numerous. Besides the mechanisms mentioned above, it has been shown in mouse β-cells in culture that addition of hydrogen peroxide causes oxidative damage that can contribute to the progressive deterioration of β-cell function (Kimoto et al., 2003).

D-Glyceraldehyde increases reactive oxygen species in rat islets via non-mitochondrial pathways, leading to oxidative stress that can be a mechanism of defective β-cell function (Takahashi et al., 2004). Disrupted cell-to-cell adherence in islets works specifically in the same direction through the action of membrane-disrupting islet amyloid polypeptide (IAPP) (Ritzel et al., 2007).

During estimation of cell apoptosis in culture using the TUNEL method, it is possible to establish not only apoptosis of β-cells, but apoptosis of all exocrine and endocrine cells as well. It is known that the process of isolation is not efficient enough to yield highly purified (approximately 100%) islet culture. Because of its existence, an apoptosis of other cells may influence functional capability of the

islets, i.e., their insulin secretion response. Besides the described case of *in vitro* conditions, it may be presumed that the same situation occurs under *in vivo* conditions.

After taking 1 ml of culture for apoptosis analyses, the remainder of the culture volume was used for stimulation at high and low glucose concentrations. The insulin secretion capacity (ISC) is cited as values per single culture, not per number of pancreatic islets, since the number of islets does not influence ISC. Basal insulin secretion during the culture period does not influence the value of ISC because the cells were centrifuged, washed, and put in a new medium before stimulation.

Apoptosis was not estimated at the beginning of the isolation process, since such an approach should decrease the yield of cell. We again point out that volume of the fetal pancreas in the gestational age used by us is low. In our experiment, the average weight of one pancreas was 0.476 ± 0.216 g. Our intention was to perform simultaneous examination of apoptosis and stimulation of β-cells in order to investigate direct influence of apoptosis on insulin secretion from islets of the fetal pancreas in culture.

Acknowledgment — It is herewith affirmed that none of the authors had any support or relationship with any company, so there is no conflict of interest. The work was supported by grant No. 145088 from the Ministry of Science and Technology, Republic of Serbia.

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АПОПТОЗА ХУМАНИХ ФЕТАЛНИХ ОСТРВАЦА У КРАТКОТРАЈНОЈ КУЛТУРИ

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У овом раду је био испитиван утицај краткотрајне културе *in vitro* на појаву апоптозе хуманих феталних панкреасних острваца (HFI) и њен утицај на масу и инсулински секреторни капацитет бета ћелија. Нађено је да је апоптоза присутна од завршетка култивације, повећавајући се у функцији времена и доводећи до смањења масе бета ћелија. У исто време ISC је био смањен. Смањење масе и ISC бета ћелија у краткотрајној култури може значајно да утиче на исход трансплантације HFI у дијабетичара са типом 1 болести.