Expression of nestin and chromogranin in regeneration zones of rat pancreas.

Ann Ital Chir, 2017 88, 1: 76-81
pii: S0003469X17026240
free reading: www.annitalchir.com

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AIM: *The particular signals that start and orchestrate the regeneration process in pancreas are not well understood yet. We aimed to investigate the expression of nestin and chromogranin A in pancreatic regeneration zones and a secondary objective, we assessed the efficiency of pancreatic duct ligation method in creation of a pancreatic regeneration model in rats.*
MATERIALS AND METHODS: *Partial (90%) pancreatectomy and pancreatic duct ligation were performed in Wistar rats, in order to create pancreatic regeneration models. Pancreatic tissues were examined histologically. Expression profiles were investigated by immunohistochemistry for nestin and chromogranin A.*
RESULTS: *Nestin and chromogranin A expressions were observed in regeneration zones. Pancreatic regenerations zones were seen in pancreatic duct ligation group samples as well as partial pancreatectomy group. Nestin was expressed prominently in acinoductular metaplasia cells in regeneration zones. This was best demonstrated in the samples of pancreatic duct ligation group. In the subsequent sections of nestin positive sites, cytoplasmic positivity with chromogranin A was observed.*
CONCLUSION: *This study confirms that nestin and chromogranin A can be detected in neogenesis-evoked pancreatic tissue, particularly in the acinoductular epithelium. Nestin and chromogranin A may be important markers to identify pancreatic stem cells. Pancreatic duct ligation can be used for creating pancreatic regeneration model in rats.*

KEY WORDS: Chromogranin A, Nestin, Pancreas, Regeneration, Stem cells

Introduction

Pancreatic islet cell transplantation is a therapeutic treatment for selected patients with type I diabetes. However, donor availability and selection and engraftment are the major obstacles in the further development of this therapy.[1] Localization and excision of suitable functional tissue is required for successful islet cell transplantation. Consequently, researchers are interested not only in the developmental stages of pancreatic cell differentiation but also in the cellular processes within mature tissues. Well-defined techniques for producing large amounts of insulin-secreting cells, originating from stem or facultative stem cells, will be highly required in the near future. Moreover, there is no consensus on either the type or origin of cells responsible for tissue regeneration in the pancreas. The primary mystery awaiting discovery is which particular signals start and orchestrate regeneration, in other words, the mechanism of regeneration. As far as we know, pancreatic stem cells exist, and they are similar to embryonic cells and have proto-differentiation skills [2,3].

Pervenuto in Redazione Luglio 2916. Accettato per la pubblicazione Ottobre 2016
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The fact that reliable stem cell markers have not been clearly identified is the underlying reason for the difficulty in identifying pancreatic stem cells [3,4]. In the literature, several proteins have been claimed to be stem cell markers, such as tyrosine hydroxylase, glucose transporter 2, cytokeratin 20, high-affinity nerve growth factor, and PDX-1. Besides these, nestin is a well-defined neuronal stem cell marker protein that can be expressed in the islet cells and rarely in ductal cells in the pancreas.[5] In this study, we aimed to investigate the expression of nestin and chromogranin A (CgA) in pancreatic regeneration zones to identify their potential role as a pancreatic stem cell marker. In addition, we assessed the efficiency of the pancreatic duct ligation (PDL) method in the creation of a pancreatic regeneration model in rats.

Materials and Methods

The study protocol was approved by local ethics and animal welfare regulatory committee (Project number: 81-10635) before it began and conformed to the ethical guidelines of the 1975 Helsinki Declaration. The study was carried out in the animal laboratory of Gazi University Faculty of Medicine.

Eight healthy male Wistar rats aged 3–5 weeks and weighing 125-128 g were used in this study. All rats were fed with a standard rat diet and regular drinking water ad libitum. The environment was arranged for 12-h day and 12-h night period at a temperature of 25°C. The rats were randomly divided into two groups. Each group included four rats and was named after the primary surgical procedure: Partial pancreatectomy (PDx) group and pancreatic duct ligation (PDL) group. Each of the groups was further divided into two subgroups. Subgroups of the PDx group were PDx-4 and PDx-7. On the fourth postoperative day, the rats were sacrificed and tissue sampling was performed in the PDx-4 subgroup. The same procedure was performed on the seventh day in the PDx-7 subgroup. Similarly, subgroups of the PDL group were designated as PDL-4 and PDL-7. All the animals were weighed daily.

Surgical Interventions

Intramuscular ketamine hydrochloride (50 mg/kg) (Ketalar®, Parke-Davis Pty. Ltd., Caringbah, NSW, Australia) was administered preoperatively for anesthesia, and surgical interventions were performed under sterile conditions. Abdominal hair was trimmed, and the skin was cleaned with 10% povidone-iodine solution preoperatively in all rats. Midline incisions were made. Abdomen closures were performed using 3/0 polypropylene sutures (Prolene®, Ethicon Inc., Somerville, New Jersey, USA) after injecting 1 cc of 0.9% NaCl (Eczaciba i-Baxter, Istanbul, Turkey) intraperitoneally. All the rats were fed with a standard diet and regular drinking water at ad libitum postoperatively. Blood glucose levels of the rats were measured before and after all interventions. The rats were sacrificed by intracardiac blood aspiration. All specimens were fixed with 10% formaldehyde solution for 6 h at 4°C and then paraffinized and blocked. Cross sections of 3-mm thickness were cut from these blocks. Prepared cross sections of the tissues collected from all procedures were stained with hematoxylin–eosin. Cross sections of the neighboring regeneration zones were selected for immunohistochemical staining.

Partial Pancreatectomy

Partial pancreatectomy was performed according to the technique previously described by Bonner–Weir,[6] and the tail and body of the pancreas were removed. The resected material consisted of splenic, gastric, and duodenal portions of the rat pancreas (approximately 90% of the pancreas). The remaining pancreas consisted of 1-2 mm^3 of the tissue left between the primary bile ducts and the first part of the duodenum. Severe bleeding did not occur in any of the rats, and anatomic structures of other organs and primary vascular structures were preserved (Fig. 1a). Sacrificing and tissue sampling were performed on the fourth and seventh days after partial pancreatec-

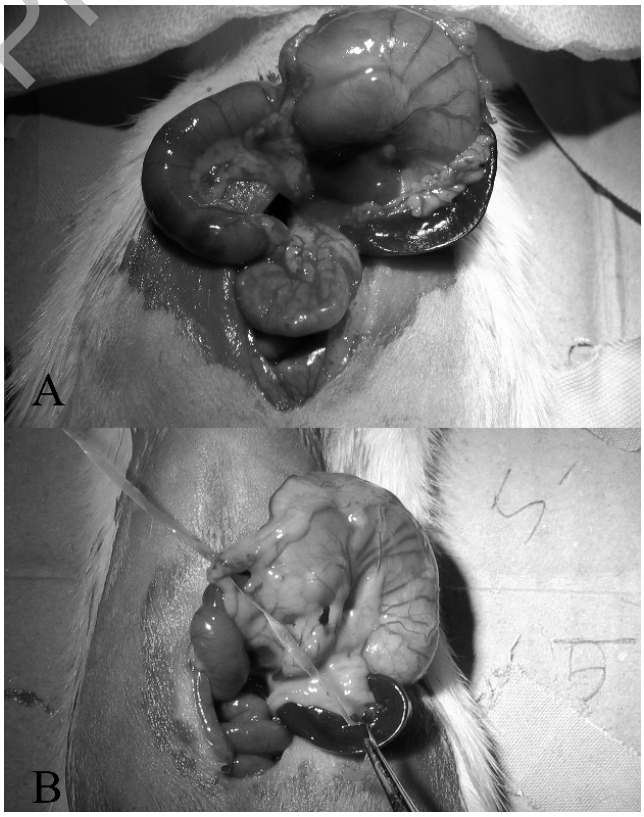

Fig. 1: A) Partial pancreatectomy; B) Pancreatic duct ligation.

tomy. Animals in the PDx-4 subgroup were operated on the fourth day. Pancreatectomy site was explored and the remaining pancreatic tissue was removed along with the duodenum. Similar procedures were performed in the rats in the PDx-7 subgroup on the seventh day.

Pancreatic Duct Ligation

Pancreatic gastric lobe of all the four rats in the PDL group was ligated with a nonabsorbable tape to ligate the gastric pancreatic duct (Fig. 1b). On the fourth day postoperatively, laparotomy was performed in the PDL-4 group. The pancreas was visualized, and the gastric lobe was resected. Then, a surgical tape was used to ligate the duct of splenic pancreas. On the third postoperative day of gastric lobe resection, laparotomy was repeated and the remaining pancreatic tissue was removed along with the duodenum before sacrificing. On the other hand, laparotomy and gastric lobe resection were performed on the seventh day in the PDL-7 subgroup. Pancreatectomy and sacrificing were performed on the eleventh day in this subgroup.

Immunohistochemical Analysis

Monoclonal rat anti-nestin antibody (MAB353B®, EMD Millipore, Massachusetts, USA) diluted at 1/50 was used in the study for nestin immunohistochemical staining. Paraffinized sections were deparaffinized for 5 min in methanol containing 3% H_2O_2 and then dehydrated. They were then washed with phosphate-buffered physiological saline solution. Nonspecific antigens were blocked by washing the sections with rabbit serum for 20 min. Primary antibodies were applied to the prepared specimens for 1 h at room temperature. After washing the preparation with phosphate-buffered saline, biotinylated anti-rat IgG was applied for 30 min. This was followed by peroxidase-marked streptavidin application for another 30 min. Peroxidase was expressed by using 3% H_2O_2 containing diaminobenzidine tetrahydrochloride. Nuclei were stained with Mayer's hematoxylin. Nestin immunohistochemical staining was performed with a standardized commercial kit. Using the same method, the tissues were stained with anti-human CgA antibody (Zymed Laboratories Inc., California, USA) and examined for endocrine differentiation.

Results

The mean blood glucose level was 210 ± 5 mg/dl postoperatively in the PDx group. The mean glucose levels of both groups were statistically similar ($p > 0.05$). Pancreatic regeneration responses were histologically *similar in all the groups.*

Light Microscopy

Histological findings were similar in all the groups. The cross sections revealed edema in the pancreatic lobular and interlobular spaces. Neutrophil infiltration was detected in the acinary interstitial zones. Dilatation and loss of granules were observed in the acinary cells. Focal regeneration zones were divided into fibrotic sections, and they consisted of duct-like cells (tubulary complexes), besides acinary cells that had dilated lumens. Acinoductular proliferation sites were observed near the pancreatic tissue, which consisted of normal acinary cells and blood vessels. Cells forming duct were cuboidal cells with eosinophilic cytoplasm. Acinoductular metaplasia sites divided by dense fibrotic tissue were observed.

Immunohistochemical Staining

Nestin was expressed in the islet cells of normal pancreatic zones and mesenchymal cells in the connective tissue. Nestin expression was not observed in normal pancreatic acinary cells or in any of the duct cells in any group. Nestin was evidently expressed in the acin-

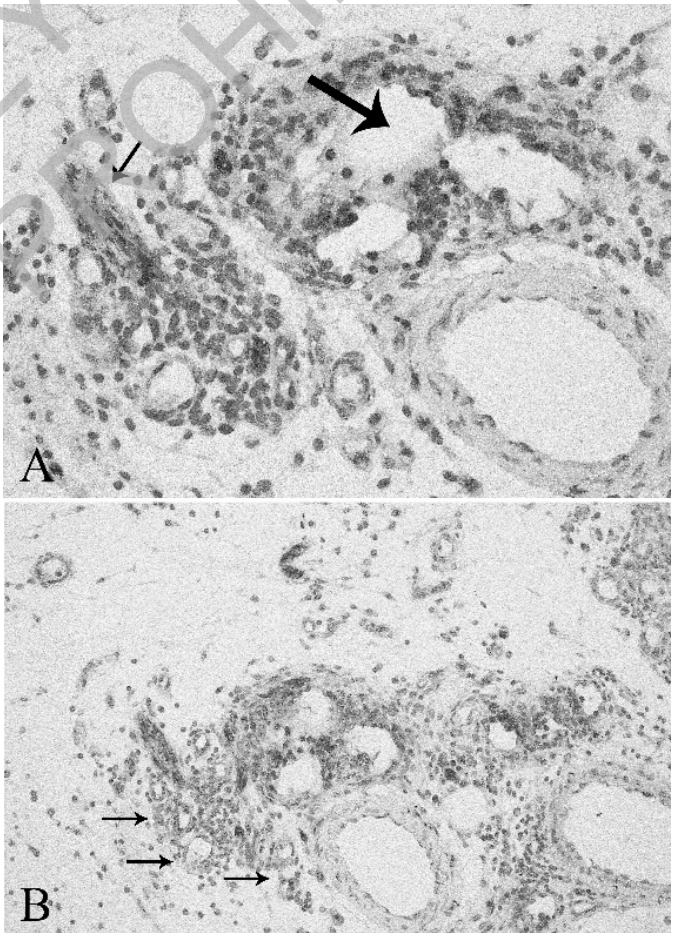

Fig. 2: A) Nestin was highly positive in the duct epithelium of acinoductular metaplasia sites (big arrow) and neighboring neuronal tissue (small arrow); B) Duct showing low-level cytoplasmic staining with nestin.

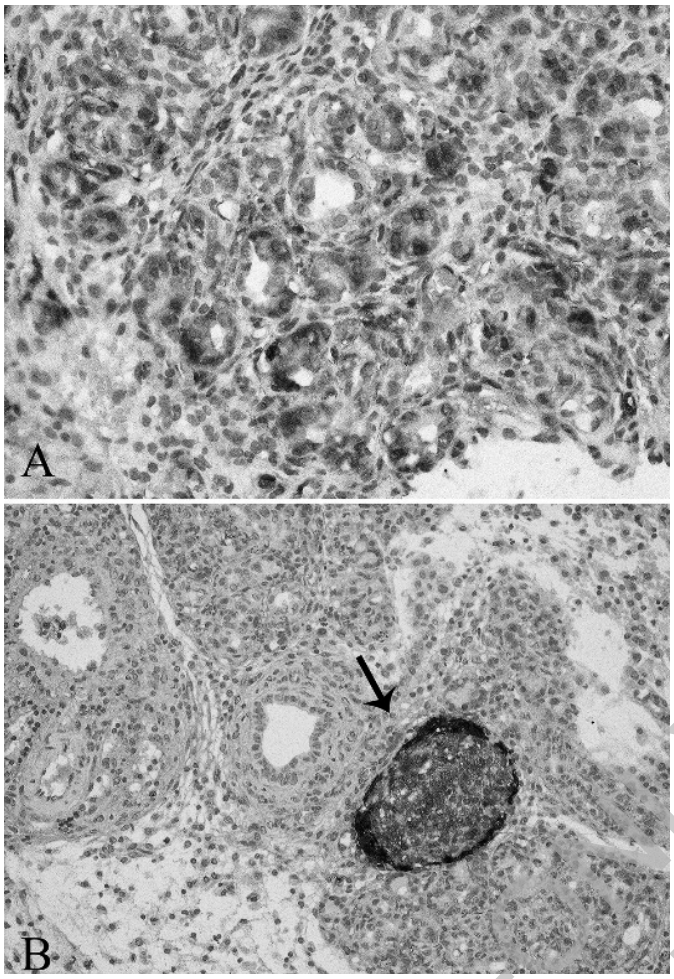

Fig. 3. A) Nestin was found to be positive in the ductal structures on day 4 in the PDx-4 group; B) Diffuse highly strong cytoplasmic positivity with chromogranin A.

oductular metaplasia cells of regeneration zones (tubulary complex cells). This was best demonstrated in the PDL-7 subgroup (Fig. 2a). Furthermore, some duct cells also had low-level cytoplasmic staining with nestin (Fig. 2b). Similarly, nestin was expressed in the regeneration centers in the PDx-4 subgroup, on the fourth day after 90% pancreatectomy (Fig. 3a).

In the regeneration zones, in the subsequent sections of nestin-positive sites, highly diffuse strong cytoplasmic positivity with CgA (mostly in the periphery) was detected (Fig. 3b).

Discussion

Transdifferentiation is a well-defined mechanism that plays a role in tissue regeneration [7]. Transdifferentiation is a proper term to describe the cell-based changes. Another term known as transformation is widely used for describing the malign transformation of normal cells. On the other hand, in metaplasia, cells do not differentiate into conventional histological structures, and the term "metaplasia" is defined as changes in the cell type of mature cells (terminally differentiated cells). During this phenotypic alteration, cells undergo dedifferentiation, proliferation, and redifferentiation [7]. The cells that are in the middle stage of dedifferentiation do not resemble the original cell phenotype, neither do the differentiated cell types. These cell types have been shown in different tissues also. In another process known as direct transdifferentiation, cells undergo transdifferentiation without proliferation and these cells have mixed phenotypes in the middle stage [8].

β-Cell neogenesis in the pancreatic tissue continues in the postnatal period [9]. Following this discovery, adult pancreatic tissue contains stem or progenitor cells [2-4]. Islet cell regeneration occurs with endocrine cell differentiation and proliferation from ductal epithelium due to the release of transcription factors during embryonic evolution. Dedifferentiated cells have been observed in the focal regeneration zones of rats that had partial pancreatectomy in experimental studies [10]. These dedifferentiated cells are pancreatic stem cells that could differentiate into phenotypic cells by external stimulation or morphogenesis. As reliable stem cell markers have not been defined because of cell characteristics, the pancreatic stem cell is difficult to be identified. After the discovery of general stem cell markers, these markers were also considered to reveal pancreatic stem cell markers. Studies conducted using these markers have demonstrated that pancreatic ductal system is important for stem cells.

Pour et al., in 1978, had demonstrated the growth of new islet cells bound to ductular cell hyperplasia and hypertrophy in their study using a carcinogen N-nitrosobis (2-oxopropy) amine [11]. They proved that new β cells migrate to the periphery from dense centroacinary cell region and they histologically resemble the growing islet cells. During this process, the acinary structure was replaced with either hyperplastic duct cells or newly formed islet cells. These new findings were important as they have not only proved that pancreatic regeneration can occur in adulthood, but they have also provided clues about the origins of islet cells.

Soria stated that nestin is an important marker for revealing stem cell differentiation of insulin-secreting cells [12]. Nestin is a known marker of neuroepithelial stem cells. It is an intermediate filament protein and expressed in the early period of tissue regeneration of different organs. Lumelsky et al., isolated nestin-positive embryonic stem cells from monkey and reported that these cells form insulin-secreting islet cells, structurally and functionally resembling pancreatic islet cells [13]. Zulewski et al., in 2001, isolated nestin-positive stem cells from adult pancreas and reported that these could be assumed as stem cells for insulin-secreting cells [5]. These studies show,

besides embryonic tissues, that nestin can be used as a stem cell marker of pancreatic stem cells that differentiate into insulin-secreting cells in mature pancreatic tissue [5,12,13]. However, the presence of nestin-positive duct cells is still a controversial issue, because some studies claim that nestin is expressed by mesenchymal cells, and not pancreatic epithelial cells [6,14,15]. Nevertheless, there are reliable studies that reported a high positivity of nestin in mesenchymal and epithelial cells [16,18].

In our study, the regeneration zones were created with neogenetic stimulation triggered by partial pancreatectomy or PDL. In these regeneration zones, nestin-positive cells were observed in the tubulary complex units. Typical ductular features were observed in the cells near the nestin-positive cells. These cells were assumed to be pancreatic stem cells that developed from the acinary or ductal system. Immunohistochemical examination of the samples collected from mature rats that underwent PDx and PDL procedures showed that CgA staining was also observed in the nestin-positive cells.

Our study demonstrated that in mature rats, PDL, which is a relatively easy-to-perform procedure than PDx, may be an appropriate way to create a pancreatic regeneration model. Procedures that stimulate pancreatic neogenesis, such as PDx and PDL, could create regeneration zones consisting of tubulary complexes. These regeneration zones contain acinoductular metaplasia cells that act as pancreatic stem cells. These cells show endocrine differentiation locally and express CgA, besides nestin, which is also suggested to be a stem cell marker.

Rosenberg et al., using a partial obstruction model of primary pancreatic duct observed some cells migrating from small intralobulary duct epithelium, which formed new islet-like structures [19]. Bonner-Weir has demonstrated that when 90% pancreatectomy is performed in mature rats, the duct cells lose their ductal phenotypes, proliferate, and turn into multipotent cells that gain potential to differentiate into islet cells under proper stimulus [6]. Under physiological conditions, these cells are repressed by local stromal factors such as transforming growth factor-β [20]. When isolated from their stroma, ductal cells show overproliferation in primary culture and differentiate into islet cells that respond to glucose.

In partial pancreatectomy and PDL models, pancreatic regeneration is triggered by the following aspects: 1. prereplicated, differentiated endocrine and exocrine cells and 2. new pancreatic lobules that differentiate and proliferate from the ductal epithelium. Regeneration zones that have ductular proliferation similar to the regeneration zones found in other animal experiments were observed in our study. These findings support the secondary pathways described in pancreatic regeneration. In other words, proliferation and differentiation occur in acinoductular cells.

All these data indicate that the pancreatic regeneration process continues in mature tissues and that acinary/ductal transdifferentiation and/or dedifferentiation play an important role in this mechanism. Understanding this mechanism will not only enlighten the pathogenesis, diagnosis, and treatment of diseases that have high morbidity and mortality, such as cancer and chronic pancreatitis, but will also be very important for autologous β-cell transplantation that could result in a significant outcome in treating diabetes mellitus.

Conclusion

In our study, the proliferation and differentiation of endocrine and exocrine cells were observed in young male rats that underwent 90% pancreatectomy and PDL. Nestin is a neuronal stem cell marker expressed in the islet cells and rarely in duct cells. In this study, nestin was found to be positive in neogenesis-evoked pancreatic tissue, especially in the acinoductular epithelium. These findings suggest that nestin could be important in identifying pancreatic stem cells, and the role of nestin-producing acinoductular metaplasia sites in pancreatic regeneration should be investigated using additional techniques.

We observed that the development of regeneration zones containing ductular proliferation can be induced by both partial pancreatectomy and PDL models in rats. Endocrine and exocrine cell proliferation and differentiation were observed in young male rats that underwent partial pancreatectomy and PDL. Such proliferation and differentiation are suggested to be orchestrated by precursor cells in the ductal epithelium. This study confirms that nestin and CgA can be detected in neogenesis-evoked pancreatic tissue, particularly in the acinoductular epithelium. Nestin and CgA may be important markers to identify pancreatic stem cells. Further investigations with larger study groups are needed for a detailed understanding of these markers.

Riassunto

Non sono ancora ben compresi i segnali di inizio e di organizzazione dei processi rigenerativi nel pancreas. È stato nostro scopo indagare l'espressione della nestina e della cromogranin A nelle zone del pancreas in corso di rigenerazione, e come obiettivo secondario quello di valutare l'efficacia del modello di rigenerazione pancreatica nel ratto ottenibile con la legatura del dotto pancreatico.

Abbiamo eseguito una pancreatectomia parziale, del 90%) e la legatura del dotto pancreatico in ratti Wistar per creare due modelli di rigenerazione pancreatica. I tessuti del pancreas sono stati esaminati istologicamente, ed i profili di espressione di nestina e cromogranina A sono stati studiati con immunoistochimica.

Sono state osservare le espressioni di nestina e cromogranina A nelle zone di rigenerazione pancreatica rilevate sia nel gruppo delle legature duttali come pure nel

gruppo delle pancreasectomie parziali. Nestina è risultata espressa in maniera preminente nelle cellule di metaplasia acino-duttulare nelle zone di rigenerazione. Ciò è stato meglio dimostrato nei casi del gruppo delle legature duttali. Nelle successive sezioni dei siti positivi per la nestina, è stata osservata una positività citoplasmatica della cromogranina A.

Questo studio conferma che nestina e cromogranina A possono essere dimostrate nel tessuto pancreatico stimolato alla neo-genesi, e particolarmente nell'epitelio acino-duttulare. Nestina e cromogranina A possono rappresentare markers importanti per l'identificazione cellule pancreatiche staminali. La legatura del dotto pancreatico può essere utilizzato per creare nel ratto un modello di rigenerazione pancreatica.

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