Biomed. Papers 148(2), 229–232 (2004)
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THE OCCURRENCE OF C-MYC, P53 AND BCL-2 FAMILY PROTEINS IN THE EARLY PHASE OF DEVELOPMENT OF DUODENAL EPITHELIUM

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Received: September 20, 2004

Keywords: Bcl-2 family/p53 family/Myc/Apoptosis/Embryo/Duodenum

In last few years, numerous groups of proteins participating in the regulation of cell proliferation, differentiation and death during ontogenesis have been described. In this study we compared the occurrence of Bcl-2, p53 and myc protein families with the level of proliferative activity and apoptosis during development of duodenal epithelium. Paraffin embedded tissues of eight human embryos and foetuses aged from the 6th – 18th week of IUD were used. For the detection of apoptotic cells the TUNEL method was performed, the proliferative marker PCNA and all the proteins studied were detected by means of indirect three-step immunohistochemical method.

In the 6th and 8th week of intrauterine development we observed isolated TUNEL positive epithelial cells only and this was accompanied by the disperse presence of PCNA as well as by all the studied proteins: Bcl-2, Bax, Bcl-XL, c-myc, N-myc, p53, p63 and p73. In the early foetal period of duodenal development we registered changes in PCNA and TUNEL positivity in accordance with the constitution of the stem cell pool on base of villi, where more numerous Bcl-2 positive cells were also found. The separation of primitive crypts and villi was not accompanied by any differences in distribution of Bax, Bcl-XL, c-myc, N-myc, p63 and p73 proteins between those compartments: all the studied proteins showed dispersed character. P53 rapidly decreased in this period. In the 18th week of intrauterine development the balance between proliferation in crypts and apoptosis of villi epithelium was well established and no p53 positive cells were found. In the presence of Bcl-2, Bax, Bcl-XL, p63 and p73 we did not find any dramatic changes. The myc proteins were restricted within the epithelium of the Lieberkűhn crypts only.

INTRODUCTION

In this study we focused on the regulation of proliferation and apoptosis by Bcl-2, p53 and myc proteins during early phases of duodenal epithelium development. Differentiation of epithelial elements in human duodenum begins quite early: between the 5th and 6th week of intrauterine development and it depends on epithelial-mesenchyme interactions[1]. After beginning of differentiation of specific intestinal epithelium and formation of villi, the separation of two different compartments in epithelium follows. This is observable in adult intestine as well: tops of villi are represented by differentiated cells whereas proliferating stem cells remain on their base. The establishment of both compartments is proceeded in human foetuses during the first trimester[2], although formation of tubular Lieberkühn crypts with typical Paneth cells at their bottom was described later, after the 14th week of development.

In the normal adult intestinal mucosa, there is a well known balance between proliferation of stem cells in Lieberkühn crypt and programmed cell death via apoptosis of terminally differentiated enterocytes on the top of villi. Although regulation of cell proliferation and differentiation is established at the end of first trimester, the appearance of first apoptotic cells in the small intestine seems to be related to definitive organization of the intestinal crypt-villus axis similar to the state of adult intestine[3]. Rare apoptotic cells were also described in earlier stages of development, where their occurrence is connected to vacuolisation of epithelium during formation of intestinal villi[4]. Less information is available about apoptosis between the 8th and 10th week of intrauterine development, which is probably the critical period in establishment of the proliferating pool of stem cells in the duodenum.

Concerning the regulation of cell differentiation and death, besides locally specific signals, the involvement of several protein families both in adult and developing intestine has been described. There is evidence for the involvement of several proteins of the Bcl-2 family in intestinal development from the 10th week of intrauterine development[5]. These proteins appear in adult intestine, as well. On the other hand, the role of p53 in intestine was studied especially in the adult intestine for its ability to block the cell cycle and induce apoptosis in cells exposed to DNA damaging stimuli[6]. However, this protein is also involved in control of cell differentiation, as was proved in cell cultures[7] and several organs[8]. This function of p53 seems to be partially influenced by the splicing variants of the rest of the p53 family proteins p63 and p73 in adult tissues[9]. The transactivating function of p53 is also involved in the control of bcl-2 gene transcription[10]: p53

enhances transcription of the pro-apoptotic bax gene and inhibits transcription of its anti-apoptotic relation gene Bcl-2. Myc proteins being able to control cell cycle, differentiation and apoptosis were found to be necessary for normal development on knock-out animal models: mice lacking either c-myc or N-myc die before the 12th day of intrauterine development and the occurrence of those proteins has been described in several parts of gastrointestinal tract[11].

MATERIAL AND METHODS

In our study we examined the duodenum of 8 embryos and foetuses aged from 6 to 18 weeks of i.u.d. All the samples were fixed in buffered formol or methacarn and embedded in paraffin in a routine method. Paraffin sections 6 μm thick were used both for labelling of apoptotic cells via the TUNEL method and immunohistochemical detection of all the proteins under study: Bcl-2, Bcl-XL, BAX, c-myc, N-myc, p53, p63, p73 and proliferative marker PCNA.

For the TUNEL method "In Situ Cell Death Detection Kit" (Roche) was used, vizualisation was performed by alkaline phosphatase-NBT/BCIP reaction. Indirect standard three-step immunohistochemical method using monoclonal and polyclonal antibodies was applied for the detection of PCNA, p-53 and Bcl-2 family proteins. For the immunohistochemical assay we used the following primary antibodies: monoclonal anti Bcl-2 (Biogenex), Bax (Immunotech), c-myc, N-myc (Santa Cruz Biotechnology) and PC-10, DO-7 and p-73 α 1.1 (all made by MOÚ) and rabbit polyclonal: p-63 α (MOÚ). Either horseradish peroxidase-DAB or alkaline phosphatase-NBT/BCIP reactions were used for vizualisation and hematoxylin or nuclear red were additionally used for counterstaining. Additionally, the sequential double staining technique was applied for several samples using the vizualisation techniques described above. We evaluated changes in protein levels in differentiating epithelium of duodenum. The occurrence of positive cells was evaluated semiquantitatively: 0 – no positive cells, + isolated positive cells, ++ regular positivity in groups of cells, +++ massive positivity.

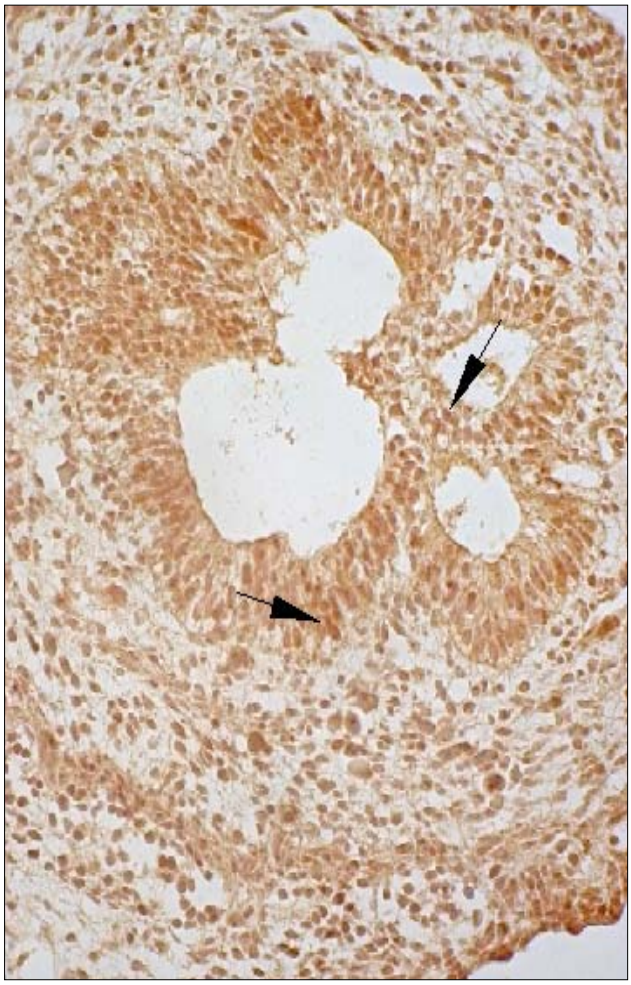

Fig. 1. c-myc positive cells (arrows) in duodenum of the 6-week-old embryo. Magn. 400x

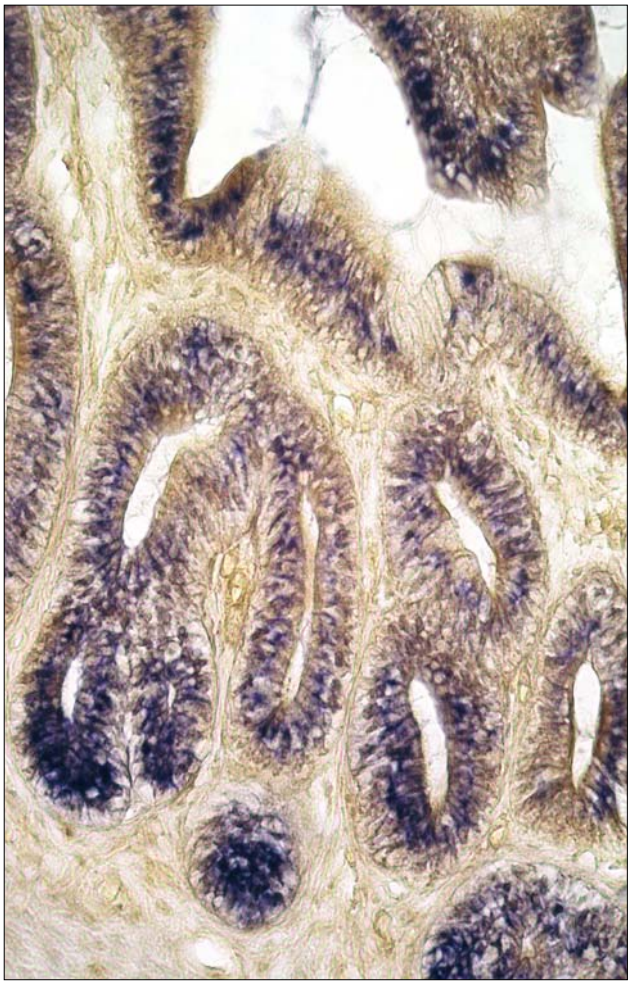

Fig. 2. Double-staining for p63 (brown) and Bax (blue) proteins in duodenum of 18-week-old foetus – detail of the crypt. Magn. 400x.

RESULTS

The 6th-8th week of IUD: We found dispersed PCNA positive cells (++) and rare TUNEL positive cells (+) with no difference in arising villi. All the studied Bcl-2 family proteins were detected: Bax and Bcl-2 positive cells were more numerous (++) than Bcl-XL (+). p53, p63 and p73 proteins were observed (++), as well as c-myc and N-myc regularly (++) in all samples in this period (c-myc occurrence shown on Fig. 1).

The 10th – 14th week of IUD: On the base of villi with pseudostratified epithelium, there were more numerous PCNA positive cells (++) in comparison to the top of villi (+) covered by single columnar epithelium. The increasing tendency of TUNEL positivity was proved especially on the top of villi. Bcl-2 distribution was similar to that of proliferation. It was present on the base of villi only (++), showing no positivity on their top. The rest of Bcl-2 family proteins, BAX and Bcl-XL did not show significant difference in distribution throughout epithelium. We also found changes in the distribution of p53 family proteins: positivity of p53 protein rapidly decreased and more positive cells were found in villi epithelium. Both p63 and p73 remained at the same level as in embryonic period with no difference between the villi bottom-top distribution. Both c-myc and N-myc proteins were present in this period and similarly to p63/p73, they did not show significant differences in their distribution.

The 18th week of IUD: This stage was characterized by strong positivity of the proliferative marker PCNA (+++) in developing Lieberkűhn crypts with no proliferation in villi epithelium. Apoptotic (TUNEL positive) cells were observed especially in villi epithelium (++), although there were found single apoptotic stem cells in crypts, too. In the distribution and number of Bcl-2 positive cells, we did not observe any changes in comparison with the previous stage. Bcl-XL and Bax positive cells were more numerous being prevalent (+++) in Lieberkűhn crypts (see Fig. 2). None p53 positive cells were found in this period, although p63 (Fig. 2) and p73 positive cells were invariably present both in crypts (++) and villi (++). Both c-myc and N-myc proteins remained only in crypts epithelium (++), no positivity was shown in villi.

CONCLUSIONS AND DISCUSSION

We demonstrated the occurence of Bcl-2, Bax and Bcl-XL proteins from the earliest periods of epithelial differentiation of the duodenal mucosa which was followed by low apoptosis. This finding was also confirmed by other authors[4]. The presence of the Bcl-2 family proteins suggest their probable involvement in interactions controlling apoptosis in embryonic stages of development albeit other important participants in this processes have not been described yet. Our observations of foetal duodenal epithelium are in accordance with studies performed on both animal[12] and human[5] tissues in the case of Bcl-2 family proteins, but not to those studying the presence of apoptosis. The presence of apoptotic cells was proved by several authors since the 18th week of development[3].

The presence of c-myc and N-myc proteins were proved from the earliest stages as well as the other proteins in this study. We did not observe any difference between localization of both proteins described by other research groups, which is dependent on the restriction of c-myc to proliferating stem cells only, and the presence of N-myc in differentiating cells, respectively[13]. In the early foetal period both proteins were localized in both epithelial departments: proliferating (villi bottom) and differentiating (villi top), later on (in the 18th week of intrauterine development), they were limited into the formed Lieberkühn crypts.

Information on the involvement of p63 in normal cell differentiation of enterocytes in adult intestine is ambiguous[14] and[15]. However, we proved both p63 and p73 in epithelium of all samples under study. Although, we cannot estimate the involvement of p63 and p73 proteins in the cell cycle/differentiation/apoptosis regulation due to the use of antibodies to their active transactivating isoforms only, our results do suggest possible involvement of this family in prenatal differentiation of the small intestinal epithelium, which may be the starting point for the next study. The presence of p53 restricted to the earliest stages of development complies with its lower importance during prenatal development[9] as well as with previous descriptive studies[16].

ACKNOWLEDGEMENT

This work was supported by grants MŠMT 15100001.

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