Acta Histochemica xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Apoptotic pathways and stemness in the colorectal epithelium and lamina propria mucosae during the human embryogenesis and carcinogenesis

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ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form 20 August 2016
Accepted 23 August 2016
Available online xxx

Keywords:
Embryo
Colon
Oct-4
AIF
caspase-3

ABSTRACT

Aim: Programmed cell death is essential both during normal organ development and carcinogenesis. In this study we immunohistochemically analyzed different pathways of cell death in 11 human conceptuses 5th-10th-weeks old, 10 low and high grade colorectal carcinomas (CRC), and 10 normal colon samples by using markers for apoptosis (caspase-3, AIF, TUNEL), proliferation (Ki-67) and stemness (Oct-4).
Results: Between the 5th and 10th week of development, caspase-3 and AIF showed moderate-to-strong expression in the developing gut wall. During development, number of caspase-3-reactive cells decreased, while AIF increased. While healthy colorectal control and low grade CRC showed moderate expression of caspase-3 and AIF, in high grade CRC their expression was strong. Tumor tissues displayed significantly higher number of positive cells than controls. Occasionally, co-expressing of both markers characterized dying cells. In developing colon, Oct-4 and Ki-67 showed moderate-to-strong expression, while some cells co-expressed both markers. Their number decreased in the epithelium and increased in the connective tissue in later development. Healthy colorectal control displayed moderate Ki-67 and mild Oct-4 reactivity. While in low-grade CRC expression Oct-4 and Ki-67 was moderate, in high-grade CRC their expression was strong. Although Oct-4 and TUNEL occasionally co-expressed in all samples, both grades of CRC contained cells that were Oct-4 positive only.
Conclusion: Our study revealed two different parallel pathways of cell death, with characteristic increase of AIF-mediated apoptosis when compared to caspase-3, and presence of stemness cells both during colon development and carcinogenesis. These finding might be considered as important diagnostic, survival and CRC therapy predictors.

1. Introduction

Colorectal adenocarcinoma (CRC) is one of the most common malignancies, with rapidly increasing prevalence and the second highest incidence among cancers, it is also the second most common cause of cancer-related mortality (Bosetti et al., 2011). The existence of many identified carcinogens and variable genetic backgrounds makes difficulty to determine most important factors in the development of CRC. The intestinal epithelium is in a constant interaction with the adjacent mesenchyme to direct stem cell proliferation, differentiation, and cell death (Potten et al., 1997). Initiation of CRC appears with the loss of control over proliferation and migration of colon crypts (Potten et al., 1997). Impairment of these processes can cause the progenitor cells to exhibit malignant properties. Usually, a histological tumor grading of adenocarcinoma is based on glandular formation (Fleming et al., 2012), thus well and moderately differentiated cancer is considered a low grade (50% gland formation), while poorly differentiated is defined as high grade CRC (less than 50% gland formation) (Compton, 2000). For CRC, surgical resection of the tumor is the first therapeutic action with curative intention. However, approximately 50% of patients

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http://dx.doi.org/10.1016/j.acthis.2016.08.004
0065-1281/

develop metastasis, which is incurable with available treatments (Khan et al., 2014). Although the survival rate has increased due to a novel chemotherapy agents and monoclonal antibodies, the overall survival has a plateau at 2 years (Cunningham et al., 2010). Additionally, good markers for predicting the biological behavior of CRC are not available and therefore the time of relapse is difficult to determine. Therefore, one of the challenges in CRC treatment is to develop new strategies to prevent the disease spreading and improve survival (Cunningham et al., 2010).

The apoptotic machinery and stemness in colon cancer is a potential avenue for investigational tools to predict aggressive behavior (Huerta et al., 2006; Koelzer et al., 2015). In cancer cells, the ability and propensity to undergo apoptosis is important in determining drug sensitivity or resistance (Zhang et al., 2007). Caspases, play a major role in apoptosis by promoting: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Igney and Krammer, 2002; Jiang and Wang, 2004), which is associated with mitochondrial outer membrane permeabilization. The family of Bcl-2 proteins is the main regulator of the mitochondrial death pathway, and can either suppress or promote mitochondrial changes. Mitochondrial pathway is mediated via p53 gene, which up-regulates apoptotic and anti-apoptotic factors of Bcl-2 family, leading to formation of pores in the outer mitochondrial membrane (Elmore, 2007) and release of AIF and other apoptotic mediators from the mitochondria (Elmore, 2007). The extrinsic apoptotic pathway occurs with the binding of apoptotic ligands to death receptors (de Jong et al., 2001; Shamimi-Noori et al., 2008). Both pathways lead to the execution of apoptosis by the cleavage of caspase-3, which results in DNA fragmentation that can be observed by TUNEL method (Cohen, 1997; Hirata et al., 1998). The detection of caspase-3 immunohistochemically could therefore be a valuable method for identifying apoptotic cells in tissue sections, even before all the morphological features of apoptosis occur (Cohen, 1997; Duan et al., 2003). Recent studies suggests that the apoptosis-inducing factor (AIF) is a caspase-3 independent cell death effector that triggers apoptosis when translocated from mitochondria to the cell nucleus (Natarajan and Becker, 2012; Ott and Herrmann, 2010). AIF is a phylogenetically ancient mitochondrial intermembrane flavoprotein, capable to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation when added to purified nuclei (Daugas et al., 2000). In addition, AIF can also participate in the regulation of apoptotic mitochondrial membrane permeabilization (Daugas et al., 2000). In response to some apoptosis stimuli, AIF translocates to the cytosol and the nucleus and cause DNA fragmentation and cell death (Cande et al., 2002b).

Oct-4 (Octamer-binding transcription-factor-4), is a central regulator of pluripotency (Nichols et al., 1998) that can be used as a stemness marker (Samardzija et al., 2012). Expression of Oct-4 is often associated with the undifferentiated cell phenotype of normal and tumors tissues (Looijenga et al., 2003). It also plays an important role in the embryonic stem cells renewal and pluripotency (Shi and Jin, 2010). Therefore, it is often used as a marker of immature and tumor cells (Kellner and Kikyo, 2010). Impairment of Oct-4 expression can cause disturbances in the process of cell differentiation (Hochedlinger et al., 2005). So far, Oct-4 has been investigated in experimental animal models, and was shown to be involved in differentiation and in tumorigenesis (Shi and Jin, 2010; Wang et al., 2013).

Ki-67 proliferative index has a prognostic and/or predictive value in different tumor types, however in colorectal cancer the results seem to be conflicting (Aoki et al., 1998; Ishida et al., 2004; Kimura et al., 2000; Kyzer and Gordon, 1997; Ofner et al., 1996; Palmqvist et al., 1999). Namely, Ki-67 cannot be correlated with the clinic and pathologic parameters that define CRC (location, age, sex, degree of differentiation, etc.), although a strong statistically significant correlation can be found with development of metastases (Aoki et al., 1998; Ishida et al., 2004; Kimura et al., 2000; Kyzer and Gordon, 1997; Ofner et al., 1996; Palmqvist et al., 1999). Therefore, Ki-67 has a limited usage as discriminatory prognosis factor in CRC. Recent studies established the fact that an increased expression of Ki-67 indicates a better survival in rectal and recto-sigmoid cancer (Salminen et al., 2005). In rectal cancer, increased proliferative activity tumors have a better response to radiotherapy, as the irradiation destroys preferentially the quickly dividing cells, while the cell population with a low proliferation activity presents an increase in the radioresistance (Menezes et al., 2010).

Table 1
Age and number of the human conceptuses analyzed in this study.

Age (weeks)	CRL (mm)	Carnegie stage	No.
5	8	15	3
8	14	17	4
10	21	20	4

Kirchner et al. proposed that appropriate arrangements of cell and tissue structures attained during embryogenesis might be associated with carcinogenesis (Kirchner and Brabletz, 2000). Namely, the patterning of neoplastic tubules during colon carcinogenesis, resemble the first patterning of a tubule in the primitive gut during embryogenesis (Kirchner and Brabletz, 2000). Thus, invasiveness and growth in the colon neoplasms can be considered as a patterning of neoplastic tubules. Wasan et al. described enlargement of micro-adenomas in human familial adenomatosis polyposis and in the multiple intestinal neoplasia in mouse by elevated rates of crypt fission (the process for the new crypt formation during the colon development) (Wasan et al., 1998). Similarly, carcinoembryonic antigen that is normally produced in gastrointestinal tissue during fetal development and is absent from healthy adult colon, is usually present in colon adenocarcinoma (Duffy, 2001). Furthermore, during development mutation of the homeobox gene Cdx2 leads to heteroplasias in the pericaecal region of $Cdx2^{+/-}$ mice (Bonhomme et al., 2003). Although intestinal expression of Cdx2 is normally present, it diminishes in CRC, suggesting its role in tumorigenesis (Bonhomme et al., 2003). Additionally, potent carcinogenic signals could be overridden by embryonic microenvironment (Pierce and Wallace, 1971). However, although many studies investigated CRC proliferation and differentiation (Nakamura et al., 1993), the link between embryogenesis and carcinogenesis remains poorly understood. Different apoptotic pathways and stemness properties could link a basic phylogenetic process of colon development which appears in embryogenesis and can reactivate again in carcinogenesis. To test this hypothesis we analyzed the apoptotic and stemness markers in the colorectal adenocarcinoma in comparison to their expression during the primitive gut development. Understanding the molecular mechanism of these processes may contribute to cancer prevention, early diagnosis and effective treatment.

2. Materials and methods

2.1. Normal and tumor adult human material

Embryonic and fetal tissues were collected and processed with permission of the Ethical and Drug Committee of the University Hospital of Split (Table 1) and adhered to the tenets of the Helsinki Declaration (Williams, 2008). Amount of 15 normal colonic and tumor samples were collected from the Department of Pathology, Cytology and Forensic Medicine, University Clinical Hospital Mostar. Macroscopic examination and measurement of the tumor samples was performed. The tumor and normal tissues of colon were treated as post-mortem material with permission of the Ethical Committee of the University Clinical Hospital Mostar, in

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