

Detection of Apoptosis in Experimental Fibrosarcoma Using DNA Fragmentation and Immunohistochemical Methods

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Abstract: Fibrosarcoma is driving from mesenchimal tissue. It is one of the malign tumors and usually seen in cats and dogs however, it can be encountered in all animals. Apoptosis used for eliminating of cells which are functioning not properly. Recent studies on tumors showed that there is a connection between cancers and apoptosis. In this study, apoptosis in fibrosarcomas induced by 3-methylcholanthrene using immunohistochemical and ELISA Methods was investigated. In order to induce fibrosarcoma in rats at the beginning of the experiment animals were injected subcutaneously on the neck with 0.2 mg 3-methylcholanthrene solved in 0.25 mL sesame oil. During the experiment took between 150-210 days depending on the appearance of tumor tissue. At the end of the experiment animals were killed under the ether anesthesia and necropsy is performed. DNA fragments of tumor tissue cell were analyzed using ELISA, localisation of Bcl-2 and Bax was determined by immunohistochemical method. Immunohistochemically there was a lot of Bcl-2 and Bax positive cells. Contrary to this in control animals there was rarely. Furthermore, the absorbance of DNA fragmentation of experimental, animals was 4 times higher than that of controls. This data indicates that in the tumor tissues it is observed some cells programmed to death by apoptosis but the others not.

Key words: Apoptosis, Bax, Bcl-2, DNA fragmentation, fibrosarcoma

INTRODUCTION

Apoptosis also called programmed cell death is a mechanism that allows cells to selfdestruct when stimulated by appropriate agents. This program is activated for various reasons, e.g., hen the cell is no longer needed within the body one when it becomes a threat to the health of the organism. Apoptosis is implicated in a variety of biological systems such as embryonic development, differentiation, tissue homeostasis and defense against pathogens. Aberrant inhibition or initiation of apoptosis contributes too many diseases including cancer. Apoptotic cell display characteristic morphological and biochemical features including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Essmann, 2000; Roshal *et al.*, 2001). DNA fragmentation is one of the most influential biochemical hallmarks of apoptosis (Kano *et al.*, 1999). Apoptosis is controlled by the expression of a number of regulatory genes including *c-myc*, *p-53* and *Bcl-2*. The proteins of the Bcl-2 family play a pivotal role in the regulation of apoptosis. All

members contain at least one of four conserved domains which are present in the prototypic Bcl-2 protein and therefore are called Bcl-2 homology domains (Kerr *et al.*, 1994). A number of Bcl-2 family members have been identified in mammals: Bcl-2, Bcl-xl, Bcl-w and Mcl-1 inhibit apoptosis where as others such as Bax, Bad, Bak, Bik and Bcl-xs, activate apoptosis. The vanoucs Bcl-2 family members can dimerize with one another with one monomer antagonizing or enhancing the function of the other. In this way, the ratio of inhibitor to activators in a cell may determine to the prosensity of the cell to undergo apoptosis (Schultz and Harrington, 2003).

Apoptosis is an essential process of cell death described in all models studied so far. It plays a crucial role in tissue homeostasis and in the elimination of damaged cells. Both increased and insufficient cell death can lead to diseases (Belkacemi *et al.*, 2005). Homeostasis of cell number is the outcome of complex coordinated processes regulating cell birth, survival and death. Abnormal accumulation of cells and the development of malignant diseases may result from either an increase in the rate of cell proliferation and/or a decrease in

apoptosis. Apoptosis is a tightly regulated energy-dependent process of programmed cell death which has been shown to play a critical role in the pathogenesis of cancer (Dayan *et al.*, 2000). Fibrosarcomas are common primary mesenchymal tumours in rodents and can occur spontaneously in various rat strains with an incidence varying from <1-3%. In mice, the incidence of fibrosarcomas varies from <1-6% in several strains. Fibrosarcomas are relatively frequent in dogs and cats but are rarely reported in other domestic animals. Most of the neoplasms develop in adult and older animals and neither breed or sex prevalence has been reported (Chandra *et al.*, 1992; Wohrmann and Teredesai, 2002). In this study, we investigated apoptosis in the rats with experimental fibrosarcoma. Apoptosis was determined as showing the levels of Bcl-2 and bax expression by immunohistochemical and assay relative quantification of histone-complexed DNA fragments.

MATERIALS AND METHODS

The study involved 16 male Inbred Sprague Dawley (105-200 g b.w., 8-10 weeks old). The animals were given standard rat chow and water according to animal experimental ethics committee guidelines. All the animals were divided into two groups: in Group 1. Fibrosarcoma was induced in 8 rats by one subcutaneous injection of 0.2 mg 3-Methylcholantrene dissolved in 0.25 mL of olive oil in the dorsal skin area; Group 2. Controls or group 8 animals received subcutaneous injection of 0.25 mL pure olive oil. The animals from the experimental group were killed soon after tumor growth was observed (about 150-210 days). The tumors were extracted on the day of detection next; two wedge-shaped sections of each tumor were taken. In the control animals, only subcutaneous connective tissue sections were collected. The half of tissue sections were fixed in 10% Neutral buffer formalin and routinely embedded in paraffin for immunohistochemical. The other tissues were stored at -70°C until assessed DNA fragmentation. This study was approved by the ethical committee of the University of Adnan Menderes.

Detection of DNA fragmentation using a photometric enzyme immunoassay: The presence of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) was quantitatively determined after induced cell death via Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals, Mannheim, Germany). Samples were homogenized with an Ultrasonical homogenizer in lyses buffer for quantification of apoptotic-related DNA fragmentation; the supernatant was collected and spun for 10 min at 20,000 g. After 20 μ L the supernatant was transferred into wells of a

streptavidincoated microtiter plate and than 80 μ L immunoreagent (including monoclonal antibody, anti-DNA-POD and antihiston-biotin which react with the DNA component of the nucleosomes) was added. The lysate was vortexed at 200 rpm and incubated at room temperature for 2 h. The plates were washed to remove unbound antibodies and the amount of histone-associated DNA fragments was quantified by analysis of the POD retained in the immunocomplex using a 2, 29-azino-di (3-ethylbenzthiazolin-sulfonate) substrate which was detected photometrically at a wavelength of 405 nm.

Immunohistochemistry: Immunohistochemical staining was performed by the standard avidin-biotin-peroxidase complex technique. Tissue samples were fixed in 10% neutral buffered formalin, routinely processed (clearing and dehydration steps), embedded in paraffin blocks, sectioned at 5 μ m using a microtome (Leica RM 2135) and stained routinely with hematoxylin and eosin (H&E). For histochemical studies, replicate sections were mounted placed on poly-L-lysine coated glass slides. After 2 h incubation at 40°C, sections were deparaffinized in xylene, hydrated through graded alcohol and endogenous peroxidase blocked with 3% H₂O₂ in 70% methanol at 15 min.

The sections were washed as in step 3 for 10 min in Phosphate Buffered Saline (PBS, pH 7.3) and nonspecific protein-binding sites were blocked with 1.5% normal pig serum (Dako Cytomatin, E0431) to reduce background staining. The sections were processed for immunolocalisation of Bcl-2 and Bax using a commercially available polyclonal antibody against Bcl-2 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and a polyclonal antibody against Bax (1: 100 dilution; Santa Cruz Biotechnology) as the primary antibodies. Then, it was incubated avidin-biotin enzyme solution at 30 min and washed three times by PBS. After slides were fixed by Diaminobenzidine (DAB), 100 μ L H₂O₂ was dropped, waited 2-3 sec in Hemalum paint and than examined at microscope.

Statistics: Istatistical analysis was performed using SPSS (for Windows Release 11.5 Standard Version Copyright© Spss Inc. 1989-2001). The results were analysed statistically using independent samples test.

RESULTS AND DISCUSSION

The onset of fibrosarcoma was observed from day 150 onwards in MCA injected rat (Fig. 1). The apoptosis in these tumor and subcutaneous tissues was monitored by the expression of Bcl-2 and Bax proteins and the detection histone-associated DNA fragments (mono and

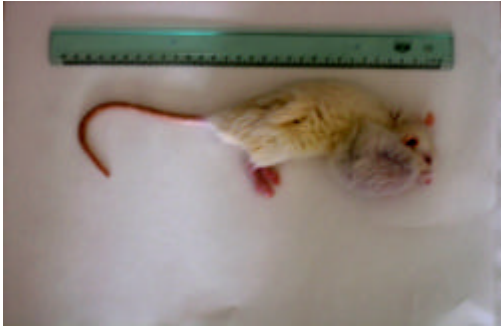


Fig. 1: MCA injected rat (282×211 mm)

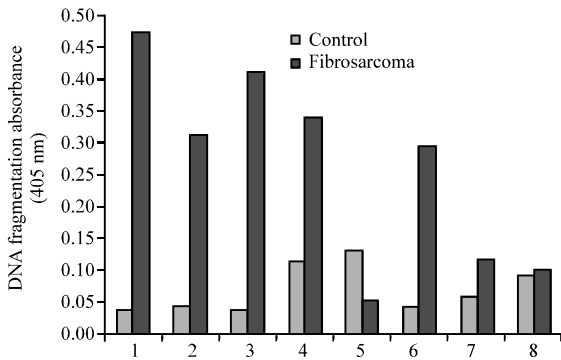


Fig. 2: DNA fragmentation in the controls and rats with fibrosarcoma ($p < 0.001$; $n: 8$). DNA fragmentation was determined using ELISA and is expressed as ELISA absorbance/mg protein

oligo-nucleosomes) released from the fragmenting nuclei of cells undergoing apoptosis by an ELISA Method. One of the hallmarks of apoptotic death is early DNA fragmentation. There was an approximately four fold increase in DNA fragmentation in tumor tissues compared the normal subcutaneous connective tissues (Fig. 2). The tumor tissues gave an ELISA absorbance of 0.262 comparison with 0.069 in control tissues ($p < 0.001$). In addition expression of Bcl-2 and Bax proteins that appeared as brown granules was identified mainly in tumor cells (Fig. 3 and 4). Subcutaneous tissues (controls) were found to be negative for Bcl-2 and Bax immunostaining (Fig. 5 and 6). Apoptosis is known to play a fundamental role in the development, the maintenance of tissue homeostasis and repair of tissues. In particular, the apoptosis machinery is essential for the ability to induce suicide of supernumerary, misplaced or damaged cells with high specificity and efficiently. The study of apoptosis has provided a significant contribution to know the physiology of several organ systems and the pathogenesis of many diseases.

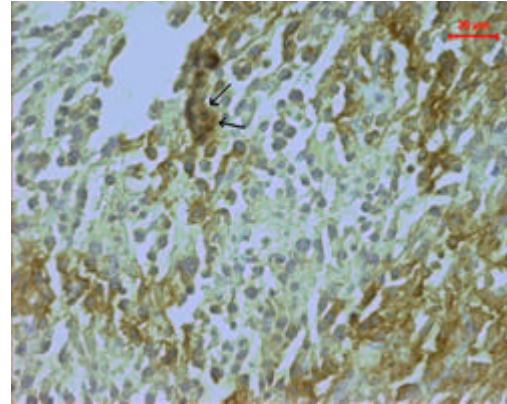


Fig. 3: Bcl-2 immunostaining positive cells in the experimental group 268×214 mm (71×71 DPI)

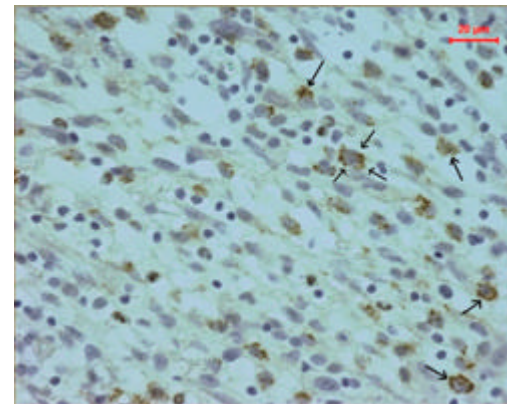


Fig. 4: Bax immunostaining positive cells in the experimental group 268×214 mm (71×71 DPI)

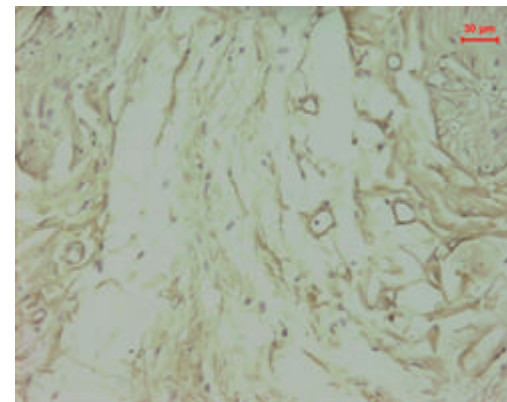


Fig. 5: Bcl-2 immunostaining negative cells in the control group 268×214 mm (71×71 DPI)

Dysfunction in the regulation or execution of cell suicide is concerned in a wide range of developmental

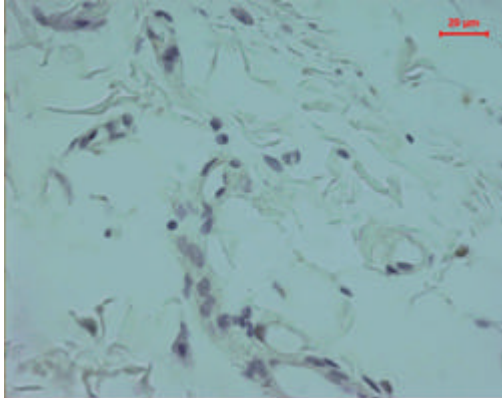


Fig. 6: Bax immunostaining negative cells in the control group 268×214 mm (71×71 DPI)

abnormalities, autoimmune and neurodegenerative diseases or cancer (Johnson, 2000; Almasan and Ashkenazi, 2003). The realization that apoptosis occurs in tumors is not new. About >20 years ago it was suggested that apoptosis may account for much of the spontaneous cell loss known from kinetic studies to occur in many tumors and it has been clear for some time that its extent often is enhanced in tumors by well-established treatment modalities such as irradiation, cytotoxic chemotherapy, heating and hormone ablation (Kerr *et al.*, 1994). In this study, we analysed the concomitant expression of Bcl-2 and Bax in normal and fibrosarcoma tissue samples. Bcl-2 inhibits apoptosis and contributes to cell survival and the resistance of cells against damaging influences.

In contrast, Bax which is considered to be a central regulator of apoptosis is a promoter of programmed cell death. The exact role of Bcl-2 in cancers is at present not clear and there is wide variability of Bcl-2 immunopositivity in different malignancies. In gastric adenocarcinomas and invasive ductal breast carcinomas, about 75% of the tumour samples exhibit enhanced Bcl-2 immunoreactivity in the cancer cells in comparison with normal tissues. In contrast in colonic carcinomas, the Bcl-2 immunoreactivity was found much lower (5% of the tumour samples) (Saegusa *et al.*, 1995). Some researchers (Gorczyca *et al.*, 1995; Wikonkal *et al.*, 1997) suggested that the presence of Bcl-2 in cancer cells seems to influence clinical outcome differently depending on the underlying malignancy. Furthermore, it was found that Bcl-2 expressed strongly in the surface epithelium of normal ovaries and benign ovarian tumors but weakly in the malignant tumors (Chan *et al.*, 2000). These findings indicated that the antiapoptotic gene *Bcl-2* was expressed in various frequencies in different cancers and a general pattern of activation or inactivation of these genes in malignant tumours cannot be defined.

Therefore, the function of apoptotic genes in different human cancers must be evaluated individually (Soini *et al.*, 1998; Hinz *et al.*, 2000). In 60 pancreatic cancers increased Bcl-2 immunoreactivity was present in 28% of the cancer samples. However, the presence of Bcl-2 in the cancer cells was not associated with the prognosis and survival time of the patients after tumor resection (Friess *et al.*, 1998). Most cancers exhibited enhanced bax expression, whereas in breast cancers bax expression in the cancer cells was found lower than normal cells in both malignancies the presence of Bax in the cancer cells is associated with longer survival compared with tumours in which Bax is absent (Bargou *et al.*, 1995; Wikonkal *et al.*, 1997). Friess *et al.* (1998) suggested that the Bcl-2-mediated antiapoptotic pathway was of minor importance for the growth behaviour and viability of pancreatic cancer cells *in vivo*. In contrast they found a significant correlation between the presence of Bax in the cancer cells and patient prognosis: increased Bax immunostaining was present in 83% of the cancer samples and its presence was associated with significantly longer survival.

CONCLUSION

The findings in present study are in agreement with the overall concept that Bax and Bcl-2 are a promoter of apoptosis and in that cancer cells, the present of Bax and Bcl-2 may be related the cell proliferation. In this study, it has been demonstrated that both Bcl-2 and Bax immunostaining were present in the fibrosarcoma cells. At that time, an early event in apoptosis is DNA fragmentation and release of nucleosomes into the cytoplasm (Salgame *et al.*, 1997). Furthermore, researchers wished to determine whether the fragmentation of DNA as detected by the ELISA, corresponded to the apoptotic process and to comparison with immunohistochemical. This procedure is sensitive and suitable for the quantitative analysis of apoptosis occurring in tissues (Gavrieli *et al.*, 1992). Therefore, ELISA which has been used successfully in kidney (Zhang *et al.*, 1997) was used for quantification of DNA fragmentation. The researchers described in their report a sensitive Enzyme-Linked Immunosorbent Assay (ELISA) for apoptosis based upon the detection of cytoplasmic nucleosomes (Zhang and Xu, 2002). This results are shown in Fig. 2. The basal value of DNA fragmentation in controls as determined by ELISA was increased approximately 4 fold in the fibrosarcoma. DNA fragmentation in apoptotic cells is followed by cell death with the dead cells being removed from the tissue within a few hours. Although, researchers expected that apoptosis decreased in experimental group

but it was increased. Considering the rapid cell proliferation of tumor tissues increased of apoptotic cells can be visualized. Therefore, additional studies should be undertaken to prognosis and elucidate the role of other proteins in fibrosarcoma's cell death.

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