Apoptosis rate in malignant mammary tumours in bitches

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SUMMARY

As the equilibrium between cell division and cell death (apoptosis) determines the growth of a tumour, the aim of the study was to evaluate the apoptosis frequency in dog mammary tumours both by the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method and by the ELISA detection of nucleosoms in cell homogenates. For that, apoptosis rates were investigated in 35 malignant tumours (16 malignant mixed tumours, 12 lobular adenocarcinomas, 3 cirrhotic adenocarcinomas and 4 myoepitheliomas) and in 10 normal mammary tissues. Whereas the apoptotic cells were found abundant and homogeneously distributed throughout the healthy breast tissues by the TUNEL method, they appeared less numerous in malignant tumours and were essentially located within mammary acini in few areas. The average amounts of nucleosoms proportional to the absorbance at 405 nm in the ELISA test in cell homogenates from malignant tumours were lower than in the healthy mammary tissues but according to the tumour type, the difference with controls was significant only for the malignant mixed tumours ($P < 0.05$). These results show that apoptosis rate tended globally to decrease in dog malignant mammary tumours, favouring in this way the selection and expansion of apoptosis-resistant neoplastic cells.

Keywords: Mammary tumour, dog, apoptosis, TUNEL, ELISA, nucleosoms.

RÉSUMÉ

Fréquence de l'apoptose dans les tumeurs mammaires malignes chez la chienne

Comme l'équilibre entre les possibilités de prolifération et de mort cellulaire (apoptose) détermine la croissance d'une tumeur, l'objectif de cette étude a été d'évaluer la fréquence de l'apoptose dans les tumeurs mammaires de chienne à la fois par la méthode TUNEL (marquage des extrémités par la terminal transférase par le dUTP-biotine) et par détection par ELISA des nucléosomes dans les homogénats cellulaires. Pour cela, le taux d'apoptose a été déterminé dans 35 tumeurs malignes (16 tumeurs mixtes malignes, 12 adénocarcinomes lobulaires, 3 adénocarcinomes cirrhotiques et 4 myoépithéliomes) et dans 10 tissus mammaires normaux. Alors que les cellules apoptotiques sont apparues nombreuses et distribuées de façon homogène au sein du tissu mammaire sain par la méthode TUNEL, elles ont semblé moins nombreuses et n'étaient localisées dans les acini mammaires que dans quelques zones. La quantité moyenne de nucléosomes proportionnelle à l'absobance à 405 nm du test ELISA s'est avérée plus faible dans les homogénats cellulaires provenant de tumeurs malignes que dans ceux issus de tissus sains mais selon le type de tumeur, la différence avec les contrôles n'a été significative que dans le cas des tumeurs mixtes malignes ($P < 0.05$). Ces résulats montrent que la fréquence de l'apoptose tend globaldement à décroître dans les tumeurs mammaires malignes des chiennes, ce qui favoriserait la sélection et l'expansion de cellules transformées devenues résistantes à l'apoptose.

Mots clés : Tumeur mammaire, chien, apoptosis, TUNEL, ELISA, nucléosomes.

Introduction

Apoptosis, a form of cell death characterized by cell shrinkage, chromatin condensation, and DNA fragmentation, can be induced by a variety of stimuli [3, 26]. Apoptosis has recently been the subject of great interest, because it has been clearly demonstrated that the cell death is not only involved during physiological development but also in neoplasia [1, 4, 11]. Cancer may be viewed as a cellular disease, in which controls that usually regulate growth and maintain homeostasis are disrupted. Normal homeostatic functioning in breast tissue is dependent on the epithelial cell turnover and on a balance between proliferation and apoptosis. Decreased cell death or excess cell division both produce hyperplasia which can be a forerunner of neoplastic transformation [22]. The exact function of apoptosis in tumour tissues has yet to be revealed [2, 6].

The detection of apoptotic cells and apoptotic bodies using normal light microscopy is based on several morphological features, including condensation of chromatin and cytoplasm and cell fragmentation which leads to the appearance of membrane-bound apoptotic bodies containing remnants of nuclei, cytoplasm, and cell organelles [23]. Due to their small size, it is difficult to detect apoptotic bodies in tissue sections using conventional light microscopy. The caspase enzymes activate endonucleases including the DNA fragmentation factor which is responsible for a highly characteristic pattern of DNA fragmentation, producing a typical DNA ladder [12, 16, 17]. Thus, apoptotic cells exhibit internucleosomal DNA cleavage and this occurred at sites between

nucleosomes (repetitive chromatin structure formed by histone association with ~200-bp DNA fragment) [8, 12]. The visualisation of endonuclease activity in tissue sections is best achieved by the TUNEL test [13]. The pathways of apoptosis play an integral part in many biological events including the removal of harmful cells, and balanced apoptosis is crucial to ensuring good health [21]. The detection of DNA fragmentation and of the presence of single strand ends of DNA is used in many studies to detect apoptotic cells [4], despite that necrosis also produces single-strand DNA ends in cell nuclei. Therefore, it is recommended to verify the results obtained using the TUNEL test by detecting cytoplasm histone-associated DNA fragments (nucleosomes) using ELISA [4].

The tumour progression may be associated with the alteration in the proliferative capacity and in the apoptosis potential of cells. These findings may help to elucidate further diagnostic and prognostic criteria [1, 20]. To the best of our knowledge, there is no data available on the rate of apoptosis in mammary tumours of bitches. Therefore, in the present study, we aimed to examine the relationship among breast carcinoma and rate of apoptosis in female dogs.

Materials and Methods

SAMPLE COLLECTION

The tumour tissue specimens were obtained during surgical operation for removal of the mammary masses and diagnosed by histopathological examinations. Forty-five samples of bitch mammary tissues were used in the present study. These included 35 malignant tumours: 12 cases of malignant mixed tumour, 16 cases of adenocarcinoma, 4 cases of myoepitelioma, and 3 cases of cirrhotic adenocarcinoma. Ten samples were obtained from normal mammary tissues. The animals were 6-16 years old and have received no treatment before the tissue sample removal.

DETECTION OF APOPTOTIC CELLS BY TUNEL

Apoptosis was detected by the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100; Chemicon International, CA, USA) [19]. The cryosections were fixed in 1% paraformaldehyde (in PBS, pH 7.4) for 10 minutes at room temperature, washed twice in PBS for 5 minutes and post-fixed in precooled ethanol / acetic acid (2 / 1) for 5 minutes at -20°C. Thereafter, the sections were incubated with 3% H_2O_2 for 5 minutes at room temperature followed by washing with PBS (2 x 5 minutes). Sections were incubated with equilibration buffer for at least 10 minutes at room temperature and then with working strength TdT enzyme at 37°C for 60 minutes, at which point the sections were incubated with working strength stop/wash buffer for 10 minutes after being agitated for 15 seconds at room temperature, followed by washing with PBS (2 x 1 minute). Sections were then incubated with anti-digoxigenin conjugate for 30 minutes at room temperature followed by washing with PBS (4 x 2 minutes). Sections were stained with 3,3'-Diaminobenzidine (DAB) solution, dehydrated, and mounted.

MEASURING OF APOPTOSIS RATE USING ELISA

The measurement of cytoplasm histone-associated DNA fragments (nucleosoms) after induction of cell death was performed with the Cell Detection ELISA plus (Manheim, Germany). Briefly, tissues were homogenised in cold lysis buffer for 3 minutes and centrifuged at 20 000 g for 10 minutes at room temperature. Cytoplasm lysates (supernatants) from control and tumour tissues were transferred to a streptavidin coated plate supplied by the manufacturer. A mixture of anti-histone-biotin and anti-DNA-POD (anti-DNA antibody conjugated with peroxidase) were added to cell lysates and incubated 2 hours at room temperature. The complex was then simultaneously conjugated with the peroxidase substrate (ABTS) to form an immune complex on the plate, whose the absorbance was read at 405 nm using an ELISA-reader (Anthos 2010).

STATISTICAL ANALYSIS

The statistical analysis of the differences between groups was determined with ANOVA, and the significance of differences with the Duncan's test. Differences were considered statistically significant when $P < 0.05$ against control group. All values were presented as mean ± standard deviations.

Results

The occurrence and the localisation of the apoptotic cells in mammary tissues were evidenced by the TUNEL test. An unequivocal TUNEL staining was seen in all of types of the tumour tissues (figure 1). Whereas in healthy control tissues the apoptotic cells were distributed throughout the breast tissue (figure 1A), they were found located in certain areas consisting mainly of acini in the most tumour tissues (figures 1B, 1C, 1D and 1E). Moreover, in some tumour tissues (malignant mixed tumours, adenocarcinomas), the number of apoptotic cells appeared diminished compared to controls. The staining pattern was mainly nuclear both in healthy and tumour tissues. A diffuse distribution or a granular reaction was seen in nuclei of apoptotic cells.

For confirming the occurrence of the apoptosis in the mammary tissues, nucleosoms (mono- and oligonucleosoms) were quantified in the cytoplasm from the mammary cells by ELISA. As reported in the Table I, the frequency of nucleosoms in cytoplasm was depressed for all the tumour tissues compared to healthy controls and it was specifically lowered in malignant mixed tumours and in cirrhotic adenocarcinomas. However, except for the malignant mixed tumours ($P < 0.05$), the difference in the apoptosis rate between healthy and the other neoplastic tissues was not statistically significant.

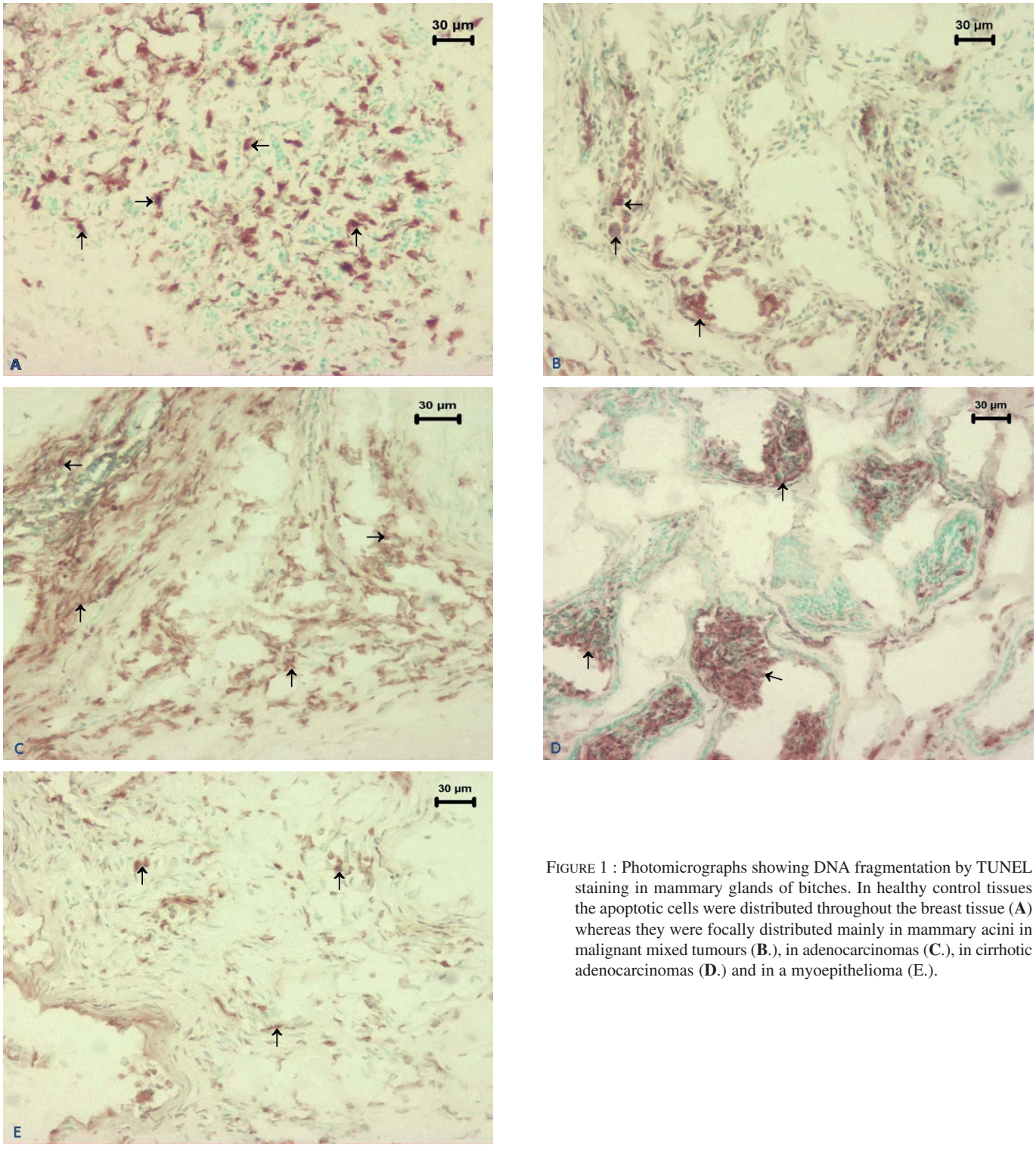

FIGURE 1 : Photomicrographs showing DNA fragmentation by TUNEL staining in mammary glands of bitches. In healthy control tissues the apoptotic cells were distributed throughout the breast tissue (**A**) whereas they were focally distributed mainly in mammary acini in malignant mixed tumours (**B**.), in adenocarcinomas (**C**.), in cirrhotic adenocarcinomas (**D**.) and in a myoepithelioma (E.).

Tissue types	**n**	**A_{405} (apoptosis rate)**
Healthy controls	**10**	**0.53 ± 0.13^{a}**
Malignant tumours	**35**	
Malignant mixed tumours	12	0.33 ± 0.20^{b}
Lobular adenocarcinomas	16	0.42 ± 0.01^{ab}
Cirrhotic adenocarcinomas	4	0.36 ± 0.03^{ab}
Myoepitheliomas	3	0.46 ± 0.02^{ab}

n: number of cases; A_{405}: Absorbance at 405 nm.
Different superscripts ^{a,b} in the same column indicate significant difference ($P < 0.05$).

TABLE I: Apoptosis rates (detection of nucleosoms in cell cytoplasm by ELISA) in the mammary glands of bitches according to the nature of the tumour. Results are expressed as mean ± standard deviation.

Discussion

Apoptosis is a continuous physiological process for the not inflammatory, programmed cell death, and is one of the today's most active fields of biomedical research [7, 10, 14, 15, 21]. As cancer results from an excessive accumulation of cells, this may be the result of an enhanced cell proliferation, a reduced cell death or both [8]. Therefore, a growing interest has been devoted to the role of apoptosis during cancer.

In the present study, the DNA strand breaks are detected by the enzymatic labelling of the free 3'-OH termini with modified nucleotides using the ApopTag® Kit. The staining intensity for healthy tissues was relatively stronger than for all of the tumour tissues. Interestingly, apoptotic cells in malignant tumours were often strongly positive. However, these positive cells were not homogenously distributed but rather located in certain parts of the tumour tissue (mammary acini) and the rest of the tumour tissue showed little even no positive staining for apoptosis. Apoptosis was evidenced in the present study by *in situ* terminal transferase-mediated dUTP nick end labelling (TUNEL). However, it is reported previously that the possibility of detection of the DNA fragmentation using the TUNEL method is very dependent on fixation and pretreatment variables [4, 19]. To verify the results obtained by the TUNEL method, an ELISA test was used for the quantification of DNA fragmentation. Absorbance at 405 nm for healthy tissues was higher than all of the tumour tissues. Among the malignant mammary tumours, the lowest absorbance was detected for the malignant mixed tumours. The higher absorbance obtained for control tissues was in accordance with the TUNEL staining pattern observed in control tissues. Although the value of DNA fragmentation in all types of tumour tissues was lower than that of control tissues, the absorbance at 450 nm was significantly lowered only in malignant mixed tumours compared to controls.

Apoptosis can be initiated by a myriad of different mechanisms in different cell types, and the kinetics of these events vary widely, from only a few minutes to several days depending on the cell system [4]. Keeping this in mind, it is possible to speculate that differences in the rate of apoptosis in different types of mammary tumours obtained in this study could be attributed to the different metabolic pathways of apoptosis. Apoptosis must be considered as a rescue mechanism that controls the integrity of the cell erasing aberrant clones and it is likely that failure of apoptosis constitutes a key factor responsible for the tumour formation and progression [9]. Changes in the expression and mutations of apoptotic proteins are common in cancer cells and contribute to the tumour development and progression [25]. The decrease in the number of apoptotic cells in malignant tumours indicates that the tumours have developed some resistance mechanisms to apoptosis. High amounts or aberrant patterns in the Bcl-2 expression have been found in a wide variety of human cancers [2, 16, 24] and the Bcl-2 protein has been proposed to be an anti-apoptosis factor, but other mechanisms for apoptosis resistance can be evoked such as P53 mutations [2, 5, 18]. The low frequency of apoptosis in the mammary tumour tissues of bitches detected in the present study indicates that tumour cells developed some mechanisms which provide these cells to escape from the primary pathway of physiological cell death. However, some cells in the tumour tissue exhibited still some apoptosis capacities and they were considered as not resistant. As they progressively disappeared by apoptosis, the cells remaining in the tumour tissues would progressively acquire apoptosis resistance and would proliferate. In this way, we confirmed here that the apoptosis pathway was blocked at least partially in the majority of the malignant mammary tumour cells in bitches and this blocking mechanism may play a pathogenic role in cancer, by favouring the clone expansion of transformed cells.

In conclusion, the apoptosis frequency in mammary tumours from bitches tends to decrease, particularly in the malignant mixed tumours. Although the programmed cell death is not a prominent feature in the tumour development, this phenomenon may contribute to select transformed cells able to resist to apoptosis and to proliferate, leading to clone expansion. However, further studies are required to identify the mechanisms for apoptosis resistance according to the tumour type in bitches. A better understanding of pathways developed by the tumour cells for surviving and for proliferating would be greatly helpful for proposing a prognostic and new therapeutic approaches based on the apoptosis up-regulation.

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