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Cancer Letters 157 (2000) 57–63

CANCER Letters

www.elsevier.com/locate/canlet

Immune modulatory potentials of antineoplaston A-10 in breast cancer patients ☆

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Received 19 March 2000; received in revised form 10 May 2000; accepted 12 May 2000

Abstract

Antineoplastons are naturally occurring cytodifferentiating agents. Chemically, they are medium and small sized peptides, amino acid derivatives and organic acids, which exist in blood, tissues and urine. Antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione) is the first chemically identified antineoplaston. Previously we have shown a strong inverse association of urinary antineoplaston A-10 with breast cancer. This study is designed to evaluate neutrophil apoptosis in patients with breast cancer at time of diagnosis and to correlate urinary antineoplaston A-10 levels with neutrophil apoptosis and to describe the direct effect of A-10 in vitro on neutrophil apoptosis in breast cancer patients. The participants were patients with a histologically confirmed diagnosis of breast cancer. Only those cases without previous treatment for breast cancer were included. Neutrophil apoptosis was assessed in breast cancer patients both morphologically and by DNA fragmentation and studied relative to healthy controls. Antineoplaston A-10 was measured using high performance liquid chromatography in urine samples collected from the patients. Urine samples from normal women served as controls. Direct effect of antineoplaston A-10 on neutrophil apoptosis was tested in vitro after adding A-10 at a concentration of 10 ng/ml to the cellular suspensions of breast cancer patients. Non-treated samples served as controls. Significantly higher neutrophil apoptosis levels were detected among patients with breast cancer with a P value <0.001. Urinary antineoplaston A-10 level is significantly negatively correlated with high apoptosis levels ($P < 0.0001$). In vitro, antineoplaston A-10 was found to inhibit significantly the neutrophil apoptosis with a P value <0.0001. These findings confirm the presence of immune defects among patients with breast cancer and such results should stimulate the development of new strategies to induce and augment immunity for the treatment of breast cancer. Antineoplaston A-10 may provide rational basis for designing trials to employ its immune modulatory potentials as adjuvant therapy in breast cancer patients.

Keywords: Breast cancer; Antineoplaston A-10; Apoptosis

1. Introduction

Cell death in a multicellular organism can occur by two distinct mechanisms, apoptosis or necrosis [1]. Apoptosis, a form of programmed cell death, is

☆ These data were partially presented at the 10th International Congress on Anti-Cancer Treatment, Paris, France, 2000.

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0304-3835/00/$ - see front matter
PII: S0304-3835(00)00472-9

becoming recognized as an important mechanism by which many cell populations and their functions are controlled [2]. Apoptosis has distinct features which include compaction of chromatin against the nuclear membrane, cell shrinkage with preservation of organelles, detachment from surrounding cells and nuclear and cytoplasmic budding to form membrane-bound fragments, known as apoptotic bodies, which are rapidly phagocytosed by adjacent parenchymal cells or macrophages [3]. Apoptosis takes place during emberogensis, in the course of normal tissue turnover and after withdrawal of atrophic hormone from its target tissue. Examples of apoptosis in adult somatic cells include formation of keratinocytes, shedding of the intestinal lining, atrophy of the prostate after castration, regression of lactating breast after weaning and the death of mature neutrophils [4]. Apoptosis is essential in the homeostasis of normal tissues of the body, especially those of the gastrointestinal tract, immune system and skin. It has become clear that regulatory mechanisms controlling programmed cell death are as fundamental and as complex as those regulating cell proliferation. Perturbation of signaling cascade regulating apoptosis can result in a wide variety of human diseases, notably cancers. There is increasing evidence that the processes of neoplastic transformation, progression and metastasis involve alterations in the normal apoptotic pathways. Failure of cells to die in response to premalignant damage allows the progression of the disease and maintains the resistance of cancer cells to cytotoxic therapy [5].

Agents that promote or suppress apoptosis can manipulate the disrupted balanced rates between cell generation and elimination that occur in cancer. Similarly, they can control cell survival and cell death pathways during a productive immune response against cancer. Neutrophils play a pivotal role in tumor immunology [6]. Neutrophil apoptosis seems to be a critical event in cancer development [7]. Therefore, a potential target in cancer treatment is to manipulate this event.

Antineoplastons are naturally occurring cytodifferentiating agents. Chemically, they are medium and small sized peptides, amino acid derivatives and organic acids, which exist in blood, tissues and urine. Antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione) is the first chemically identified antineoplaston [8].

This study is designed to evaluate neutrophil apoptosis in patients with breast cancer at time of diagnosis to determine if difference in immunity exists between breast cancer patients and control, to correlate urinary antineoplaston A-10 levels with neutrophil apoptosis and to describe the direct effect of A-10 in vitro on neutrophil apoptosis in breast cancer patients.

2. Materials and methods

2.1. Patients and controls

The participants were women between 30 and 67 years of age who had a histologically confirmed diagnosis of breast cancer. Only those cases without previous treatment for breast cancer were included. All of the age-matched controls had no history of cancer or any other breast diseases.

2.2. Preparation of blood neutrophils

Neutrophils were isolated from peripheral blood of patients and healthy controls by a combination of dextran sedimentation and centrifugation through discontinuous plasma percol gradients as described by Haslett [9]. Briefly, freshly drawn venous blood was citrated (4.4 ml of 3.8% sodium citrate to 10 ml blood) and centrifuged at 3000 rev./min for 20 min at 20°C. The plasma was aspirated and divided into two volumes, one was centrifuged at 2500 rev./min for 10 min for production of platelet poor plasma (PPP) and the second volume was calcified by adding 20 mM of calcium chloride to prepare platelet rich plasma derived serum (PRPDS). To the red and white cells remaining in each tube, 5 ml of 6% dextran (500 000 mol wt) in 0.9% saline were mixed gently and then allowed to stand for 30 min for sedimentation of erythrocytes. The leukocytes were aspirated, centrifuged at 2750 rev./min for 6 min, resuspended in 2 ml PPP and the suspension was underlayered with 2 ml of 42% percol in PPP, then followed by 2 ml of 51% percol in PPP. The neutrophils were then aspirated from the interface of the 51 and 42% percol.

2.3. Culture of neutrophils

Neutrophils prepared as described above were resuspended in an appropriate volume of RPMI

1640 medium with 10% autologous PRPDS and 100 μl/ml of penicillin and streptomycin. The suspension in culture tubes was incubated at 37°C in 5% carbon dioxide. The age of neutrophils in the culture was calculated at the start of culture at time zero (base line), 24, 48 and 72 h.

2.4. Assessment of cell viability

At time zero and then at subsequent times, cells were removed from culture and counted on a hemocytometer. Cell viability was determined by Trypan Blue dye exclusion test.

2.5. Assessment of neutrophil apoptosis

The apoptotic neutrophils in culture were assessed both morphologically and by agarose gel electrophoresis.

2.5.1. Morphological assessment of apoptosis

At time zero and after 72 h, cells were removed from each culture, harvested on slides, fixed in methanol, and stained with:

1. May Grunwald Giemsa [10] and examined by oil immersion light microscope. Five hundred cells/slide were examined. Neutrophils were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, condensed and fragmented nuclei, decrease cell size, loss of membrane integrity and cytoplasmic vaculation [11].
2. Acridine orange stain (AO). One drop of cell suspension was added to one drop of AO solution (10 μg/ml in phosphate buffer saline), mixed gently on a slide, and immediately examined with an Olympus BH-2 microscope with fluorescence attachment. The produced green fluorescence was detected between 500 and 525 nm. Cells exhibiting bright fluorescent condensed intact or fragmented nuclei were interpreted as apoptotic cells [12].

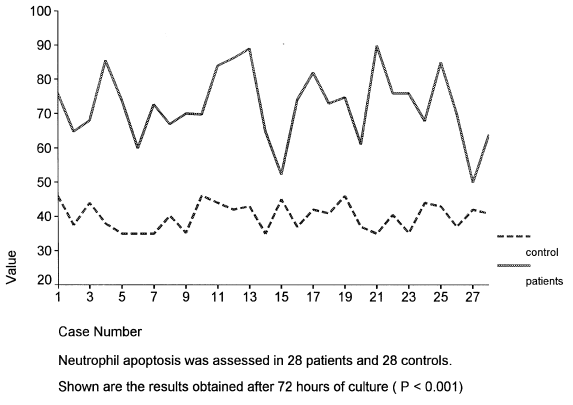

Fig. 1. Neutrophil apoptosis levels in breast cancer patients relative to control.

2.5.2. Agarose gel electrophoresis for DNA fragmentation assay

Assessment of chromatin fragmentation of neutrophils was done by modification of methods previously used for thymocytes [13]. Cells were lysed by adding 0.5 ml of cell suspension to lysis buffer [4.5 ml of Tris–HCl buffer (pH 8.0), 20 mmol of EDTA and 0.2% Triton X-100]. After standing for 1 h, the lysate was centrifuged at 3500 rev./min at 4°C for 20 min to separate high from low molecular weight chromatin. The supernatants were collected into tubes and precipitated with one volume of 5 m mol/l of NaCl and two volumes of absolute ethanol. The DNA was precipitated for 24 h at 40°C. The precipitate was centrifuged at 2500 rev./min at 6°C for 15 min. The pellet was resuspended in l ml of 10 m mol/l of Tris–HCl buffer (pH 8) containing 100 m mol/l EDTA and 0.1 m mol/l of sodium dodecyl sulfate. Proteinase K was added to a final concentration of 20 μg/ml and the sample was incubated for a further 24 h at 37°C. The DNA was extracted with phenol and chloroform and reprecipitated with absolute ethanol. The pellets were dissolved in 20 μl of lysis buffer and 10 μl of RNase. Each sample of the purified DNA (20 μl) was subjected to electrophoresis in 1% agarose gel containing 30 μg/ml ethidium bromide and was visualized under ultraviolet light. Size of fragments were confirmed by reference to a 1-kb DNA ladder (Gibco/BRL). Cells undergoing programmed cell death exhibited DNA fragmentation and appeared as ladder pattern [14].

2.6. Detection and determination of A-10 in urine

Urine (25 ml) was purified by Amberlite XAD-2 resin by washing with water (100 ml ×2), 100 ml

methanol:water (1:1) and finally 100 ml pure methanol. The last two fractions were evaporated under reduced pressure at 35°C. The produced residues were spotted on thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) and developed in a solvent system (1-butanol:glacial acetic acid:water; 4:1:1 v/v). The developed plates were visualized under short UV lamp. The R_f was determined as 0.63 in comparison with an authentic sample from A-10 [15]. The final residues were spotted on HPTLC and the concentration of each spot was determined by CAMAG TLC scanner (Wilmington, NC).

2.7. Effect of antineoplaston A-10 on neutrophil apoptosis in vitro culture

At time zero and according to a dose response curve (data not shown), neutrophils in culture were incubated with 10 ng/ml antineoplaston A-10. Non-treated samples served as controls.

2.8. Statistical analysis

The student *t*-test is used to examine the difference between neutrophil apoptosis in breast cancer patients relative to their healthy controls. Also, to examine the difference between neutrophil apoptosis levels in antineoplaston A-10, treated and non-treated samples of breast cancer patients. Spearman's rank correlation analysis was used to evaluate the relation between urinary antineoplaston A-10 level and neutrophil apoptosis levels. A P value of <0.05 was considered significant.

3. Results

3.1. Neutrophil apoptosis levels in breast cancer patients and controls

Apoptosis of neutrophils as assessed both morphologically and by DNA fragmentation of 28 breast cancer patients was studied relative to 28 healthy controls. Readings after 72 h of culture were recorded. Significantly higher neutrophil apoptosis levels were detected among patients with breast cancer with a P value <0.001 (Fig. 1).

3.2. Correlation between neutrophil apoptosis and urinary A-10 level in breast cancer patients

The correlation coefficients between urinary antineoplaston levels and neutrophil apoptosis levels in the same 28 breast cancer patients were estimated by Spearman's rank correlation analysis. Urinary A-10 level is significantly negatively correlated with high apoptosis levels with a P value <0.0001 (Fig. 2).

3.3. Effect of antineoplaston A-10 on neutrophil apoptosis in breast cancer patients

Neutrophil apoptosis was assessed after adding antineoplaston A-10 to the cellular suspensions of 42 breast cancer patients at a concentration of 10 ng/ml. Non-treated samples served as controls. Readings after 72 h of incubation were estimated. A-10, treated and non-treated samples when compared, antineoplaston was found to inhibit significantly the neutrophil apoptosis with a P value <0.0001 (Fig. 3).

4. Discussion

Neutrophils play a pivotal role in tumor immunology. Several reports indicate that these cells are capable of mediating the lysis of leukemic cells as well as solid tumor cells [6,16–19]. The neutrophil response to mediate tumor cell lysis includes the generation of reactive oxygen intermediates [20] and/or the release of lytic molecules prepacked in their granules [21,22]. The lysis of tumor cells by

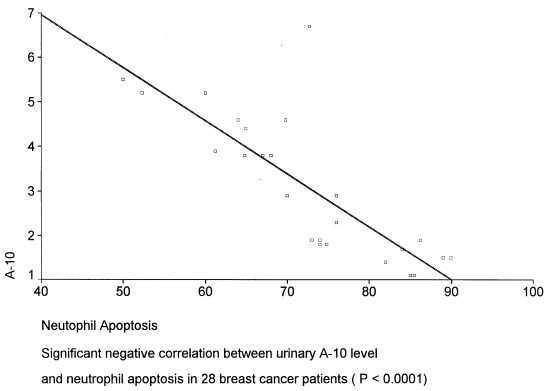

Fig. 2. Correlation between neutrophil apoptosis and A-10 level in breast cancer patients.

neutrophils also was shown to be enhanced by various cytokines [23,24].

Impaired functions of neutrophils have been encountered in different types of tumors. In patients with lung cancer, neutrophil chemotaxis was significantly lower than healthy volunteers [25] and significant alteration of neutrophil phagocytosis was found in colorectal adenocarcinoma patients at time of diagnosis [26]. In breast cancer, spontaneous TNF-alpha production by neutrophils was lower, compared with that found in healthy persons. Similarly, stimulated neutrophils of cancer patients produced significantly lower amounts of TNF-alpha compared with that produced by neutrophils of healthy controls [27]. The different abilities of neutrophils to release TNF-regulatory proteins (TNFRs) has been shown and this appears to confirm neutrophils participation in TNF-mediated reactions of the host during malignant process [28]. Furthermore, the respiratory burst of neutrophils could serve as a prognostic parameter in head and neck cancer [29].

Neutrophil apoptosis seems to be a critical event in cancer development. IL-2 maintain the viability of neutrophils by preventing these cells from apoptosis and in vivo depletion of mature granulocytes resulted in adenocarcinoma growth, thereby confirming a direct role for IL-2 activated neutrophils in tumor cytolysis [7]. Changes in apoptosis of neutrophils with aging could play a role in the increased incidence of certain immune system related pathologies of aging, such as cancer [30].

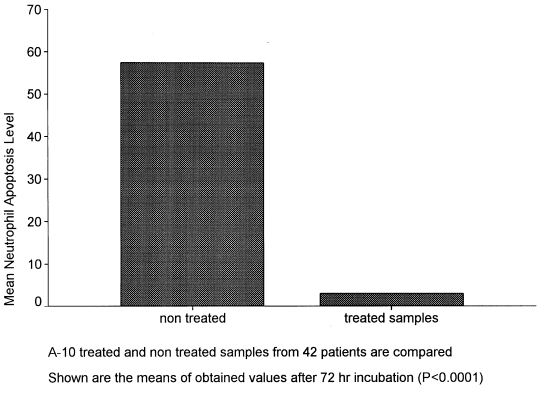

Fig. 3. Effect of antineoplaston A-10 on neutrophil apoptosis in breast cancer patients.

Apoptosis, is an active, genetically controlled process that removes unwanted or damaged cells. Suppression, overexpression or mutation of a number of genes which orchestrate the apoptotic process are associated with disease. The diseases in which apoptosis has been implicated include those with increased cell survival with inhibition of apoptosis and those with excess cell death where apoptosis is overactive. Cancer and apoptosis are related in many ways. In particular, defective apoptosis may permit the persistence of damaged or mutated cells that would otherwise have been deleted. On the other hand, apoptosis controls cell survival and cell death pathways during effective immune response against cancer. A potential target in cancer treatment is to manipulate such pathways. Our finding of high neutrophil apoptosis levels in breast cancer patients suggest that neutrophil apoptosis may be a feature of neoplasia and identify a potential new target for apoptosis-based therapy in breast cancer. Antineoplastons are naturally occurring cytodifferentiating agents. Chemically, they are medium and small sized peptides, amino acid derivatives and organic acids, which exist in blood, tissues and urine. Antineoplaston A-10 is the first chemically identified antineoplaston. The reported inhibitory effect of A-10 on various tumor cells suggests a potential benefit for the treatment of human cancers with such agent [31–35]. In the study of Tusda et al. [34], the antineoplastons were less toxic than conventional chemotherapeutic agents and they were useful in maintenance therapy for cancer patients. A-10 significantly inhibited the growth curve of human breast cancer serially transplanted to athymic mice [35]. Such activities of A-10 coupled with the low toxicity and novelty of basic A-10 structure provide an exciting possibility of developing a new class of clinically useful antineoplastic drugs with minimal side effects.

Here we describe its immune modulatory potentials. Urinary A-10 levels were significantly negatively correlated with neutrophil apoptosis. Furthermore, it significantly inhibited neutrophil apoptosis when tested in vitro. Taken together, these findings confirm the presence of immune defects among patients with breast cancer and such findings should stimulate further studies to develop the use of immunity as a potential tumor marker or as a prognostic indicator and should stimulate the development of strategies to induce and augment immunity for the

treatment of breast cancer. Antineoplaston A-10 may provide rational basis for designing trials to employ its immune modulatory potentials as adjuvant therapy in breast cancer.

References

[1] A. Eastman, S. Grant, R. Lock, T. Tritton, Cell death in cancer development, Cancer Res. 54 (1994) 2812–2818.

[2] T. Nakajima, H. Aono, T. Hasunuma, L. Nishioka, Apoptosis and functional Fas antigen in rheumatoid arthritis synoviocytes, Arthritis Rheum. 38 (1995) 485–491.

[3] G. Firestein, M. Yeo, N. Zvailer, Apoptosis in rheumatoid arthritis synovium, J. Clin. Invest. 96 (1995) 1631–1638.

[4] P.A. Hall, P.Y. Coates, B. Ansari, D. Hopwood, Regulation of cell number in mammalian gastrointestinal tract: the importance of apoptosis, J. Cell Sci. 107 (1994) 3569–3577.

[5] R.J. Bold, P.M. Termulhen, D.J. McConkey, Apoptosis, cancer and cancer therapy, Surg. Oncol. 6 (3) (1997) 133–142.

[6] P. Katz, C.B. Simon, P.A. Henkart, A.S. Fauci, Mechanisms of antibody dependent cellular cytotoxicity: the use of effector cells from chronic granolomatous disease patients as investigative probes, J. Clin. Invest. 65 (1980) 55–63.

[7] F. Pericle, R.A. Kirken, P.K. Epling Burnette, B.K. Blanchard, J.Y. Djeu, Direct killing of interleukin-2 transfected tumor cells by human neutrophils, Int. J. Cancer 66 (1996) 367–373.

[8] S.R. Bruzynski, T.L. Loo, D.H. Ho, P.N. Rao, J. Georgiades, H. Kratzenstein, Biologically active peptides in human urine, Physiol. Chem. Phys. 8 (1976) 13–22.

[9] C. Haslett, M. Guthrie, M. Henson, Modulation of multiple neutrophil functions by preparation methods, Am. J. Pathol. 199 (1985) 101–110.

[10] C.D. Gregory, C. Dive, S. Henderson, C.A. Smith, G.T. Williams, J. Gordon, A.B. Rickinson, Activation of Epstien–Barr virus latent genes protect human B cells from death by apoptosis, Nature 349 (1991) 612–614.

[11] A.H. Wyllie, R.G. Morris, A.L. Smith, D. Dunlop, Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis, J. Pathol. 142 (1984) 67–77.

[12] M. Whyte, M. Meagherr, C. Haslett, Loss of functional responsiveness to surface stimuli in apoptotic neutrophils, J. Leukocyte Bio 1 (1990) 29–32.

[13] W. Bursch, F. Oberhammer, R. Schulte-Hermann, Cell death by apoptosis and its protective role against disease, Trends Pharmacol. Sci. 13 (1992) 245–251.

[14] D.L. Green, C. Dive, Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and non-proliferating immature thymocytes, Cancer Res. 53 (1993) 2133–2139.

[15] L. Abou-Zeid, Ph.D Thesis, Antiestrogen and/or antiprogestin structurally related new drugs of chemotherapeutic value, Mansoura University, Egypt, 1995.

[16] F. Dallegri, G. Frumento, A. Ballestro, R. Goretti, F. Patrone, Relationship between antibody-dependent tumor cell lysis and primary granule exocytosis by human neutrophils, Clin. Exp. Immunol. 70 (1987) 479–483.

[17] G. Bruchelt, R. Handgretinger, A. Kimmig, B. Goeke, B. Siedner, R.A. Reisfeld, D. Niethammer, J. Treuner, Effect of granulocytes on human neuroblastoma cells measured by chemiluminescence and chromium-51 release assay, J. Biolumin. Chemilumin. 3 (1989) 93–96.

[18] B.H. Kushner, N.K.V. Cheung, GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma, Blood 73 (1989) 1936–1941.

[19] E. Barker, B.M. Muueller, R. Handgretinger, M. Herter, A.L. Yu, R.A. Reisfeld, Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells, Cancer Res. 51 (1991) 144–149.

[20] S.J. Weiss, A.F. Lobuglio, Biology of disease: phagocyte-generated oxygen metabolites and cellular injury, Lab. Invest. 47 (1982) 5–18.

[21] A.K. Lichtenstien, T. Ganz, T.M. Nguyen, M.E. Selsted, R.I. Lehrer, Mechanism of target cytolysis by peptide defensins: target cell metabolic activities, possibly involving endocytosis, are crucial for expression of cytotoxicity, J. Immunol. 140 (1988) 2686–2694.

[22] E. Barker, R. Reisfeld, A mechanism for neutrophil-mediated lysis of human neuroblastoma, Cancer Res. 53 (2) (1993) 362–367.

[23] J.M. Michon, A. Gey, S. Moutel, E. Tartour, V. Meresse, W. Fridman, J. Teillaud, In vivo induction of functional Fc gammaRI (CD64) on neutrophil modulation of blood cytokine mRNA levels in cancer patients treated with G-CSF (rMetHuG-CSF), Br. J. Haematol. 100 (3) (1998) 550–556.

[24] T. Uchida, T. Yamashita, A. Arakii, H. Watanabe, F. Sendo, rINF-gamma-activated rat neutrophils induce tumor cell apoptosis by nitric oxide, Int. J. Cancer 71 (2) (1997) 231–236.

[25] R. Shirai, J. Kadota, K. Iida, K. Kawakami, K. Abe, M. Yoshinaga, T. Iwashita, Y. Matsubara, M. Oka, S. Kohno, Immunological competence and nutritional status in patients with lung cancer, Lung 176 (6) (1998) 363–370.

[26] J. Lukac, Z. Kusic, D. Kovacevic, Z. Soldic, B. Troskot, Neutrophil and monocyte phagocytic functions in patients with colorectal adenocarcinoma during fluorouracil therapy, Anticancer Res. 15 (1995) 2805–2809.

[27] E. Jabonska, Serum levels of tumor necrosis factor and the production of this cytokine by polymorphnuclear cells in breast cancer patients, Arch. Immunol. Ther. Exp. 46 (2) (1998) 93–96.

[28] E. Jabonska, M. Kiluk, W. Markiewiccz, Soluble TNF receptors release by polymorphnuclear cells and the serum levels in breast cancer patients before and after treatment, Neoplasma 45 (1) (1998) 17–20.

[29] W. Kaffenberger, B.P. Clasen, D. Van Beuningen, The respiratory burst of neutrophils, a prognostic parameter in head and neck cancer? Clin. Immunol. Immunopathol. 64 (1992) 57–62.

[30] T. Fulip Jr., C. Fouquet, P. Allaire, N. Perrin, G. Lacombe, J. Stankova, M. Rola Pleszcynski, D. Gange, J.R. Wagner, A. Khalil, G. Dupuis, Changes in apoptosis of human polymorph-

nuclear granulocytes with aging, Mech. Ageing Dev. 96 (1997) 15–34.

[31] H. Tsuda, A. Iemura, M. Sata, M. Uchida, K. Yamana, H. Hara, Inhibitory effect of antineoplaston A-10 and AS2-1 on human hepatocellular carcinoma, Kurume Med. J. 43 (2) (1996) 137–147.

[32] Y. Sugita, H. Tsuda, H. Maruiwa, M. Hirohata, M. Shigemori, H. Hara, The effect of antineoplaston, a new antitumor agent on malignant brain tumors, Kurume Med. J. 42 (3) (1995) 133–140.

[33] H. Tsuda, S. Sugihara, H. Nishida, H. Hara, N. Eriguchi, K. Ishii, K. Sasaki, S. Yoshimura, N. Tanaka, The inhibitory effect of the combination of antineoplaston A-10 injection with a small dose of *cis*-diamminedichloroplatinum on cell and tumor growth of hepatocellular carcinoma, Jpn. J. Cancer Res. 83 (5) (1992) 527–531.

[34] H. Tsuda, H. Hara, N. Eriguchi, H. Nishida, H. Yashida, T. Kumabe, Y. Sugita, Toxicology study on antineoplaston A-10 and AS2-1 in cancer patients, Kurume Med. J. 42 (4) (1995) 241–249.

[35] K. Hashimoto, T. Koga, Y. Shintomi, M. Tanaka, T. Kakegawa, H. Tsuda, H. Hara, The anticancer effect of antineoplaston A-10 on human breast cancer serially transplanted to athymic mice, Nippon Gan Chiryo Gakkai Shi 25 (1) (1990) 1–5.