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SEMIQUANTITATIVE CYTOCHEMICAL METHOD IN THE EVALUATION OF SMOKING INDUCED CHANGES OF ALVEOLAR MACROPHAGES' GLYCOGEN AND APOPTOTIC PROPERTIES

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Having in mind hypothesis about discrete changes in alveolar macrophages (AMs') biological markers under the smoking exposure, the study was designed regarding cytological, cytochemical and apoptotic parameters in lung washings. Cell profile and apoptotic capacity (AC) of pulmonary tissue evaluated by bronchoalveolar lavage (BAL) were evaluated by light microscopy in fifteen subjects: 9 non-smokers and 6 smokers. Apoptosis was detected by TUNEL in situ cytochemical method. Semiquantitative indexing and scorring methods were used for AC and evaluation of alveolar macrophages (AMs) glycogen by PAS reaction. Significant increase of macrophages, AC and decrease eosinophils ($p<0.05$) were revealed in smokers in comparison with

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nonsmokers. There is significant correlation of AMs' glycogen and smoking exposure (Spearman R= 0.98, $p<0.001$) as well as eosinophils and AMs' glycogen (Spearman R=0.81, $p<0.05$). In nonsmokers, percentage of free apoptotic bodies (FAB), correlates with amounts of glycogen in AMs (Spearman R=-0.79, $p<0.05$). The evidence of smoking induced changes of AMs' metabolic properties supports an idea of cell energy metabolism switching which might be important for programmed cell death regulation in order to avoid harmful influence of noxious agents, including tobacco smoke.

Key words: alveolar macrophages, glycogen, apoptotic capacity, bronchoalveolar lavage, smoking

INTRODUCTION

Since the macrophages are interposed between stress, inflammation and immune response in the maintenance of basic survival response (OTTAVIANI, 1997), it is a question how they can participate in the process of apoptosis in tissues exposed to different pathogenic and cancerogenic stimuli. As the pulmonary-tissue-resident cells, AMs contribute in the clearance of tissue during the process of programmed cell death of non-resident, cells recruited from the blood to lung during pathogenic process. Changes in the metabolic status of AMs, might be represented in part, as the changes of amounts of intracellular glycogen. In physiological terms the glycogen particle represents an organelle "machine" whose function is to store glucose and make it available on demand.

As a cigarette smoke affects the main lung defense system at both structural and cellular level, it was found of interest to observe some dynamism in the process of generation of apoptotic bodies originating from mononuclear cells and their removal by resident macrophages.

Apoptosis is considered as a protective mechanism that limits lung injury (ORITZ, 1998). Within the tissues, most apoptotic bodies are rapidly phagocytized either by resident macrophages or neighboring cells and are degraded within phagosomes by lysosomal enzymes derived from the engulfing cell (HARMON, 1988).

The aim of study was to analyze incipient changes and interactions of cytological, metabolic and apoptotic parameters in pulmonary tissue under the smoking exposure.

MATERIALS AND METHODS

Patients

Fifteen patients were lavaged: 9 nonsmokers and 6 smokers. All the patients underwent bronchological unit in order to perform routine diagnostic procedures because of longstanding cough or bleeding, but without signs of lung

disease after detailed clinical and bronchological examination. Total smoking exposure in group of smokers was calculated as follows:

Pack-years = (Age of presentation-age started-years stopped) x pack/day

It was 1.5; 1.2; 1,5; 1; 0.6 and 5 for individual patients in the group of smokers. Passive smoking was not considered for non-smokers and for all examinees, total smoking exposure was 0.

Bronchoscopy and laboratory management of BAL

Bronchoalveolar lavage was performed by fiberoptic bronchoscopy. Lavage cell differential counting was performed as suggested by HASLAM (1984). Cytocentrifuge slide preparations, air dried at room temperature were made from each lavage sample for differential cell count and cytochemical analysis.

Differential cell counting in BAL

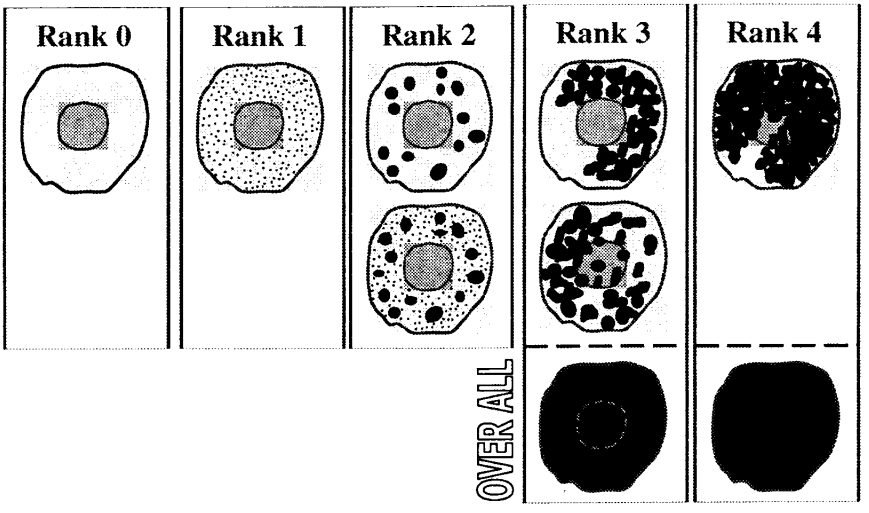
The samples were stained with May-Grunwald-Giemsa stain for differential counting and analysed under a light microscope (ZEISS, Axioplan) for at least 500 cells, regarding next cell types: alveolar macrophages, lymphocytes, neutrophils, eosinoiphils and mast cells.

Cytochemical analysis of AMs

Cytochemical analysis of amounts of glycogen (PAS reaction) in AMs' was peformed as it has described earlier by ŽUNIĆ *et al.* (1996). The intensity of PAS reaction was evaluated semiquantitatively according to the following individual cell ratings:

- Positivity grade 0 of the cytochemical reaction relates to AM with absence of stained products of cytochemical reaction in the cell;
- Positivity grade I indicates AMs with a random distribution of diffuse small granules, like dust
- Positivity grade II - granules are larger, single or in small groups, with cytoplasmic localization;
- Positivity grade III relates to AMs with large deposits of staied products in cytoplasm and/or covering the nucleus; the same grade is given to AMs with diffuse staining of cell, but low intensity;
- Positivity grade IV relates to large deposits which mask a major part of the cytoplasm, diffusely spread in the cells, which makes recognition of cellular structures impossible.

The grade of cytochemical reaction positivity has been estimated in 300 AMs. The percentage of AMs with the same grade of cytochemical positivity was calculated and then multiplied by appropriate index varying between 0-5.5, as it is schematically presented in the Figure 1. The final score for single slides was within a theoretical range between 0 and 550.



Spearman's rank rule:

Rank 0x0 + Rank 1x1 + Rank 2x2 + Rank 3x3.5 + Rank 4x5.5 = Score [0–550]

(%) (index)

$\frac{3+4}{2}$ $\frac{5+6}{2}$

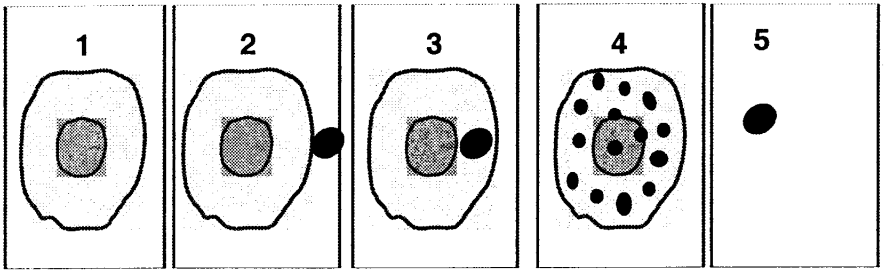
TUNEL assay

Apoptotic detection in cytocentrifuge preparations of BAL cell suspensions was evaluated by light microscopy using TUNEL in situ cytochemical method (Boehringer Mannheim, In Situ Cell Death Detection Kit, POD; Cat. No: 1 684 817) (MILOŠEVIĆ, RAKIĆ and RUŽDIJIĆ, 1998; MILOŠEVIĆ *et al.*, 2000) and modified for BAL cytocentrifuge preparations (ŽUNIĆ et al, JBRHA, in press).

Indexing and scoring method and calculation of apoptotic capacity

Property of AM to engulf apoptotic cells was estimated by light microscopy including 1000 features per sample. These features are related to subsequent steps (adsorption, internalization and digestion of apoptotic bodies by AMs). Based on the yield of each one, indexing and scoring method was performed as presented schematically in Fig. 2.

Percentage of each stage was multiplied with the corresponding index (starting from 1 to 4; FAB are not indexed - their percent is expressed separately) and the sum calculated. Then, the final score for single slide calculated was within theoretical range from 100 to 400. The score is a numerical equivalent of tissue ability for apoptosis and removal of apoptotic bodies, i.e. it represents the apoptotic capacity of the tissue.



Apoptotic capacity (AC) - relation between presence of free apoptotic bodies (FAB) and their removal by phagocytosis

1000 estimated features = Stage 1 + Stage 2 + Stage 3 + Stage 4 + FAB
(1) (2) (3) (4) (5)

Stage 1x1 + Stage 2x2 + Stage 3x3 + Stage 4x4 = Score
(%) (%) (%) (%) (AC)

Statistical analysis

Statistical analysis of the results (t-test and Spearman rank order correlation test) was performed using STATISTICA for Windows program, suitable at PC.

RESULTS

Differential cell counting, glycogen content in AMs and AC of pulmonary tissue were evaluated in fifteen examinees (9 non-smokers and 6 smokers) and results presented in Table 1.

Table 1. - Cytological and cytochemical parameters in investigated groups

	Non-smokers percent of cells Mean SD N=9	Smokers percent of cells Mean SD N=6	Non-smokers vs. smokers Mean SD
Neutrophils	2.16 ± 1.51	1.05 0.82	not significant
Eosinophils	2.54 ± 1.92	0.75 0.54	$p<0.05$
Mast cells	0.52 0.37	0.37 0.30	not significant
Lymphocytes	19.50 ± 4.58	15.90 3.76	not significant
Macrophages	65.86 5.77	75.18 ± 6.25	$p<0.02$
PAS reaction-score	514.56 36.79	513.34 32.41	not significant
AC	218.29 ± 56.24	289.55 ± 50.77	$p<0.05$
FAB (%)	1.84 ± 1.16	0.50 ± 0.38	$p<0.02$

Significant decrease of percentage of eosinophils ($p<0.02$) and increase of AMs ($p<0.05$) were found in smokers in comparison with non-smokers. There was no significant difference of scores for PAS reaction in investigated groups. There is significant correlation of AMs' glycogen and smoking exposure (Spearman $R= 0.98$, $p<0.001$) as well as eosinophils (Spearman $R=0.81$, $p<0.05$), respectively. In nonsmokers percentage of free apoptotic bodies correlate with amounts of glycogen in AMs' (Spearman $R=-0.79$, $p<0.05$).

Significant differences were obtained in apoptotic parameters, AC and percentage of FAB under the smoking influence. AC was significantly increased ($p<0.05$) and percentage of FAB decreased ($p<0.02$) in smokers in comparison with non-smokers.

DISCUSSION

Glycogen is the main carbohydrate reserve store in the majority of animal tissues. PAS- Periodic acid Schiff reaction is based at periodic acid (HIO4)-oxidizing agent. The amount of color developed by the reaction is dependent primarily on the amount of reactive glycol structure present in tissues. PAS - positive tissue compounds are: 1) polysaccharides (glycans), including glycogen; 2) glucosaminoglycans and glucosaminoglucuronglycans (sialoglycans); 3) glycoproteins and glycopeptide; 4) Glycolipids (gangliosides and cerebrosides (inositol phosphatides); 5) unsaturated lipids and phospholipids, including ceroid (Pearse, 1972). The chemical technique most extensively used in carbohydrate histochemistry is the periodic acid Schiff (PAS) reaction which is positive with structures containing neutral hexose sugars and /or sialic acids.

Theoretical basis for the present study and data evaluation was described in our previous studies where semiquantitative cytochemical analysis of AMs was performed by indexing and scoring method (ŽUNIĆ *et al.*, 1996; ŽUNIĆ *et al.*, 1997) in analysis of AMs' enzyme activity and their metabolic properties. These cells have some specific features such as big size and unique shape in comparison with other cells occurring in BAL fluid. Advantage of semiquantitative cytochemical indexing and scoring method is that the recognition of intracellular products of cytochemical reaction is relatively simple. The fact that AMs are originated from locally supplied monocytes from the bloodstream, makes the problem with dilution factor avoidable.

In the present study we examined the apoptotic capacity of the pulmonary tissue, which reflects relation between the presence of FAB (originating from mononuclear phagocytes in bronchoalveolar space, and then, in BAL) and their removal by phagocytosis by non-apoptotic AMs. This is the main difference in the approach applied here and the classical method of apoptosis quantification in tissue by evaluation of percentage of apoptotic cells (MILOŠEVIĆ, RAKIĆ and RUŽDIJIĆ, 1998; MILOŠEVIĆ *et al*, 1999). The value of AC is not the absolute parameter. It has been determined based on randomly encountered pulmonary resident and non-resident cells in their functional features during the

process of programmed cell death. As number of FAB in situ might be changed due to their increased/diminished generation and/or phagocytosis, AC is more useful parameter than apoptotic rate, in the case when clearence of apoptotic remnants is important for evaluation.

In spite of unrecognized nature of physiological and pathological events and signaling pathways in phases of cell-to-cell communication, internalization and processing of phagocytized particles, it is possible to make an approximate semiquantitative evaluation of the processes regarding their complexity. (Fig.2). For example, the process of FAB adherence to AMs is the first among processes from adherence to internalization and intracellular digestion of internalized cells and it is characterized by index, or weighing factor 2 (weighing factor, or index of 1 is for clear AMs). The second stage is more complex because the processes of adherence and internalization are considered at the same time and indexed by weighing factor 3, and the last one - the process of intracellular digestion comprises all of these above mentioned processes and additionally a new one, resulting in digestion inside the AMs. It is weighted by index 4 and represents the most complex stage, which includes at least three phases: adherence, internalization and digestion.

FAB decrease in smokers in comparison with that of non-smokers, might be in correlation with stimulated process of digestion inside the AMs what indicates a possibility of a fast FAB clearance in smokers, or phagocytosis of the apoptotic remnants as rapidly as they are generated. In NSCLC group, the processes of FAB adsorption to AMs, internalization and intracellular processing of engulfing remnants (stages 2-4) are decreased in comparison with those of non-smokers and smokers. This leads to decreased scores, or their numerical equivalent - apoptotic capacity of pulmonary tissue in NSCLC. A decreased capacity for degradation of apoptotic bodies in AMs of the patients with NSCLC could be in relation with a decreased phagocytosis of the apoptotic remnants, what can be either the cause or the consequence of oncogenic process.

The founding of significant decrease of BAL eosinophils and increase of AMs in smokers in comparison with that of nonsmokers might be understood as the consequence of changed tissue architecture of pulmonary tissue in situ. There are experimental evidences that smoking alters redistribution of lung immunocytes between interstitial changes and the development of proliferative alveolar and bronchial lesions (ZEID and MULLER, 1995). Tobacco smoke contains carcinogenig compounds responsible for DNA damage (WEST K. A. *et al*, 2003).

Smoking alters immunological competence of different cell populations in pulmonary tissue. In cigarette- smokers an alveolitis develops characterized by a 3-5-fold increase in the concentration of alveolar macrophages (AMs). Delayed apoptosis of AMs represents one of the mechanisms for accumulation of these cells. The fact that cigarette smoking causes morphological changes within the airways and forces a reconstruction of all constituents of the bronchial mucosa within 10-20 years, opens the field of diagnostic interests with the scope to reveal discreet, but still reversible alterations (SCHABERG, 2000).

Presented results reveal significant inverse correlation of percentage of free apoptotic bodies, and amounts of glycogen in AMs. Apparently, glycogen amounts are changed under the smoking influence (the evidence of significant correlation of AMs' glycogen and smoking exposure). Otherwise, the presence of significant correlation between BAL-eosinophils and amounts of glycogen in AMs in smokers, supports an idea that immune response of pulmonary tissue under the smoking depends on cell energy metabolism switching. These findings might be important for understanding of multifactorial regulation of programmed cell death in order to avoid harmful influence of noxious agents, including tobacco smoke.

In conclusion, only smoking, without other known pathological conditions causes specific regulatory pattern. Metabolic and immunological properties of pulmonary immunocytes, might be understood as possible early biomarkers of remodeling process within the bronchial epithelium.

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SEMIKVANTITATIVNI CITOHEMIJSKI METOD U EVALUACIJI PUŠENJEM IZAZVANIH PROMENA U SADRŽAJU GLIKOGENA ALVEORALNIH MAKROFAGA I NJIHOVIH APOPTOPSKIH KARAKTERISTIKA

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Izvod

Alveolarni makrofazi su uključeni u imuni odgovor u plućnom tkivu kroz niz složenih interreakcija sa drugim imunim ćelijama koje na zahtev migriraju iz krvnih sudova u tkivo. Pušenje utiče na karakteristike imunog odgovora u tkivu pluća i na proces remodeliranja tkiva. U grupama nepušača, (N 9) i pušača, (N 6) urađena je bronhoalveolarna lavaža. Citospin preparati bronhoalveolarnih lavata su bojeni May Grunwald Giemsa om za diferencijalno brojanje ćelija, pomoću svetlosnog mikroskopa. Od citohemijskih tehnika su korišćeni TUNEL, *in situ* metod za detekciju apoptoze i PAS reakcija za detekciju glikogena u alveolarnim makrofazima. Imajući u vidu fagocitne sposobnosti ovih ćelija, definisan je apoptotski kapacitet kao numerički ekvivalent sposobnosti tkiva da stvara slobodna apoptotska tela i da ih odstranjuje fagocitozom posredstvom neapoptotskih alveolarnih makrofaga. Rezultati ukazuju na povećanje prinosa makrofaga i smanjenje eozinofila u bronhoalveolarnim lavatima pušača u poređenju sa nepušačima ($p<0.05$). Iako nije nađena statistički značajna razlika sadržaja glikogena u alveolarnim makrofazima pušača u poređenju sa nepušačima, kod pušača skor za Perlsovu reakciju korelira sa ekspozicijom duvanskom dimu (Spearman $R= 0.98$, $p<0.001$) i sa prinosom eozinofila u bronhoalveolarnom lavatu (Spearman $R=0.81$, $p<0.05$). Kod nepušača, relativni procenat slobodnih apoptotskih tela korelira sa sadržajem glikogena u alveolarnim makrofazima (Spearman $R=-0.79$, $p<0.05$). Promene metaboličkih svojstava alveolarnih makrofaga, koje se reflektuju na sadržaju glikogena, mogu biti u osnovi promenjenih imunoloških i apoptotskih osobenosti tkiva pod uticajem pušenja.

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