

In vitro model for studying malignancy associated changes

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Abstract. Malignancy associated changes (MAC) can be defined as subtle morphological and physiologic changes that are found in ostensibly normal cells of patients harboring malignant disease. It has been postulated that MAC have a potential to become a useful tool in detection, diagnosis and prognosis of malignant diseases. An *in vitro* cell culture model system was designed to study interactions between non-small cell lung cancer (NSCLC) and the normal bronchial epithelium of the human respiratory tract *in vivo* to see if the MAC-like phenomenon can be detected in such a system. In this study we examined changes in nuclear features of normal human bronchial epithelial cells (NHBE) when they were co-cultured with cells derived from a lung cancer cell line NCI-H460. Using discriminant function analysis, nuclear features were determined which allow maximal discrimination between normal cells incubated with or without cancerous cells. Our results demonstrate that MAC appear to be specific to changes induced by malignancy, and that these changes differ from those induced by growth factors in the serum. This study provides evidence in support to the hypothesis that MAC are induced by a soluble factor(s) released by malignant cells.

Colour figure can be viewed on <http://www.esacp.org/acp/2003/25-2/sun.htm>.

Keywords: Cell co-culture, image cytometry, nuclear features, malignancy associated changes

1. Introduction

Malignancy associated changes (MAC) are defined as subtle morphological and physiologic changes that are found in normal cells of patients harboring malignant disease. These changes were first observed by Gruner in 1916 [10], while the term “MAC” was first described by Nieburgs in the late 1950s in buccal mucosa [20]. In both cases, these changes were found in patients with late stages of malignancy. Since then MAC have been described in many tissue types in patients with invasive cancers by several authors [7,17–19]. Nevertheless the concept of MAC was not generally accepted due to a subjective nature of its description as well as poor reproducibility of the observations by pathologists [8].

The big impetus in MAC research came with the development of image cytometry. The existence of MAC was first proven in an objective way in 1974 by Klawe and Rowinski [13], followed by Wied et al. [25] and Burger et al. [4]. Thereafter many other laboratories reported the measurements of MAC by a variety of nuclear and cellular features, all of which were consistent with subtle changes in morphology of the normal cells growing in the vicinity of a malignant growth.

It has been proposed that the phenomenon of MAC can be employed as a test for the detection and objective diagnosis of a wide variety of early cancers [21,22]. For example, we have previously demonstrated that sensitivity of the conventional sputum cytology can be improved by the use of image cytometry by utilizing MAC [23]. In that study sputum sensitivity of 80% was obtained compared to the sensitivity of 20–40% generally found by conventional sputum cytology alone [2].

Despite a broad body of research employing MAC in the detection of cancer, the causal relationship of

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malignancy to MAC has not yet been clearly demonstrated. There are two major competing postulates of the mechanisms of MAC. The first hypothesis postulates that MAC arise from a concurrent exposure of normal tissue to the environmental carcinogens which caused the neoplastic lesion. The second hypothesis postulates that MAC are caused by soluble chemical mediator(s) or factor(s) released by the malignant cells which then diffuse to the surrounding cells and affect them. We believe that evidence is accumulating that favors the latter hypothesis.

However, the mechanism of MAC remains poorly understood and relatively unexplored. For this reason we designed a defined and controlled *in vitro* model to experimentally study the underlying biological and molecular events in order to better understand MAC. The aim of this study was to develop an *in vitro* model and to test the hypothesis that MAC are induced by soluble factors released by malignant cells.

2. Materials and methods

2.1. Cell cultures

2.1.1. NHBE cultures

Normal human bronchial epithelial cells (NHBE) were purchased from Clonetics Corp. (San Diego, California) and maintained in BEGM medium (Clonetics Corp., BEBM supplemented with 0.5 ng/ml human recombinant epidermal Growth Factor, 5 $\mu\text{g/ml}$ insulin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 10 $\mu\text{g/ml}$ transferrin, 0.5 $\mu\text{g/ml}$ epinephrine, 6.5 ng/ml triiodothyronine, 50 $\mu\text{g/ml}$ gentamycin, 50 ng/ml amphotericin-B, bovine pituitary extract and 0.1 ng/ml retinoic acid). They were plated onto 0.7 cm^2 glass coverslips positioned in 24-well culture plates (Fisher Scientific, Nepean, ON, Canada) at a density 1×10^4 cells/coverslip and allowed to grow to confluency. NHBE cells were used for the co-culture experiments two days after reaching confluency.

2.1.2. NCI-H460 cultures

The NSCLC cell line (NCI-H460) was donated to us by Dr. A. Gazdar, University of Texas Southwestern Medical Center, Dallas, USA who also made available this cell line to the American Tissue Culture Collection (ATCC). This cell line is well characterized [3,9]. Cells were maintained in RPMI 1640 medium (GIBCO, Burlington, ON, Canada) supplemented with 5% FBS. For the co-culture experiments, cells were

plated on 6×2.5 cm glass slides and allowed to grow in the growth medium until they reached the log growth phase. At this stage slides were washed with PBS three times and the growth medium was changed to BEGM medium. Cells were then further cultured under these conditions for an additional period of 24 hours prior to the co-culture experiments.

2.1.3. Co-culture experiments

Co-culture experiments were carried out in 100 cm^2 square tissue culture dishes (GIBCO, Burlington, ON, Canada). NHBE cells grown on coverslips and NCI-H460 cells grown on glass slides were placed together in the dishes. For controls we used NHBE cells grown on coverslips, co-cultured with NHBE cells grown on glass slides. Cells were co-cultured for 24, 48, and 72 hours at 37°C in an atmosphere of 95% air, 5% CO_2 . The ratios of tumor cells to normal cells in the co-culture groups were 2.5 : 1, 5 : 1 and 10 : 1. These ratios were achieved by placing a different number of slides with lung tumor cell line into the petri dishes, while keeping the number of coverslips with NHBE cells constant. At the end of the co-culture incubation time, the NHBE cells were fixed with Sedfix (Surgipath Medical Industries, Inc., Richmond, IL, USA) for 30 minutes and then air dried.

2.2. Cell DNA staining

The NHBE cells were stained by the Thionin-Feulgen method described by Tezcan et al. [24]. This is a stoichiometric, DNA specific stain (Oncometrics Imaging Corp., Vancouver, BC, Canada) employed routinely in the measurements of nuclear features using image cytometry. All samples from a particular experiment were stained together in the same staining batch to reduce staining variations.

2.3. Image analysis

For each Feulgen-stained sample, digital images of the nuclei of approximately 600 NHBE cells were collected in an automated way using a high resolution image cytometer (Cyto-Savant, Oncometrics Imaging Corp.). The effective pixel size was $0.34 \mu\text{m} \times 0.34 \mu\text{m}$ (about $0.1 \mu\text{m}^2$). For each image, 126 nuclear features [6] were calculated including morphological features, photometric features, discrete texture features, Markovian and non-Markovian texture features, run-length features and fractal features. The mean of integrated optical density (IOD) value of the NHBE cells from each coverslip was used to normalize the optical

density features to compensate for any stain intensity variations between the slides. Images acquired from each sample were sorted into quality images of cell nuclei or “junk” objects (overlapping cell nuclei, out of focus cell nuclei, cellular debris) by a cell/junk classifier (binary decision tree) generated using discriminant function analysis. Quality images of cell nuclei were sorted into four groups including diploid, S-phase, aneuploid and tetraploid cells, using histograms of the nuclear DNA amount. Only diploid cells were used in all subsequent analysis.

2.4. Statistical analysis

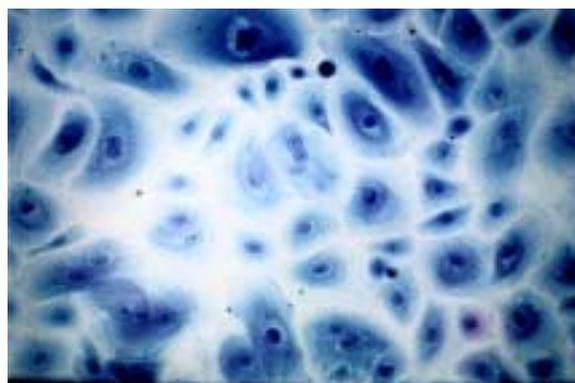
Linear discriminant function (DF) analysis (BMDP Statistical Software, Inc., Los Angeles, CA, USA) was used to derive a linear discriminant function separating the NHBE cells that were co-cultured with NCI-H460 cells from the NHBE cells that were incubated with normal cells on glass slides. In this analysis nuclear feature values of 200 diploid NHBE cells were taken from each sample; those co-cultured with highest concentration (10:1) of malignant cells were pooled into the MAC group and those co-cultured with normal cells were pooled into the normal (control) group. This process generated about 3×10^5 cells for each group that were used in statistical analysis. Cell by cell stepwise discriminant analysis was performed selecting from over 100 features describing nuclear size, shape, chromatin distribution (texture features) and DNA content. The resulted discriminant function (MAC expression) was then utilized to classify all the NHBE cells into MAC or non-MAC group in every sample, including samples, co-cultured with lower concentration of malignant cells (2.5:1, 5:1) that were not used in training. Frequency of cells expressing MAC was then calculated as the number of NHBE cells classified as MAC divided by the total number of NHBE cells in the sample. The latter feature was used to discriminate between samples.

3. Results

3.1. Visual determination of MAC

Colour figure can be viewed on <http://www.esacp.org/acp/2003/25-2/sun.htm>.

When NHBE cells were plated on coverslips and allowed to divide until a confluent monolayer was formed, they were of a polygonal shape with clearly



(a)



(b)

Fig. 1. (a) Confluent NHBE cells co-cultured with lung tumor cells (H460) in BEGM for 24 hours. (b) Confluent NHBE cells cultured in BEGM for 24 hours. Magnification: 40×10 . Papanicolaou staining. This figure can be viewed on <http://www.esacp.org/acp/2003/25-2/sun.htm>.

defined edges. No morphological changes could be observed under the light microscope (40×10) between NHBE cells cultured for 24 hours in the presence of malignant cells (Fig. 1a) or in the absence of malignant cells, but co-cultured with normal cells (Fig. 1b).

3.2. Quantitative approach to measuring of MAC

A large number of nuclear features showed significant differences in mean values and variances when group of NHBE cells co-cultured with the highest concentration of NCI-H460 cells was compared with NHBE cells incubated with controls. Stepwise linear discriminant function was trained to separate these two groups. Table 1 shows 11 nuclear features selected by the analysis to form resulted function of MAC expression.

Table 1

Eleven features, selected by the discriminant function analysis to form a linear combination which best discriminated NHBE cells expressing MAC and no-MAC group

Feature type	Features name	Feature description
Morphological feature	Harmon_03_fft	Nuclear boundary variation
Photometric features	Var_intensity	Variance of intensity
	DNA_index	Normalized DNA amount
Discrete texture features	HiDNA_area	Percentage of the nuclear area occupied by a high density chromatin
	LowVSmh_DNA	Measure of compactness of high density chromatin
	LowDNA_Comp	Average extinction ratio of medium and high density chromatin
	Med_av_dst	Average distance between medium density chromatin clumps and centre of the nucleus
Markovian texture features	Homogeneity	Measure of "smoothness" of image intensity
Non-Markovian texture features	Range_extreme	Global intensity range estimation
	Center_of_gravity	Measure of misbalance in the distribution of chromatin condensation state
Fractal texture feature	Fractal_dimension	Fractal measure of imaginary 3-dimensional surface created by the optical density function of the nucleus; gives highest values for nuclei with frequent and significant changes of optical density.

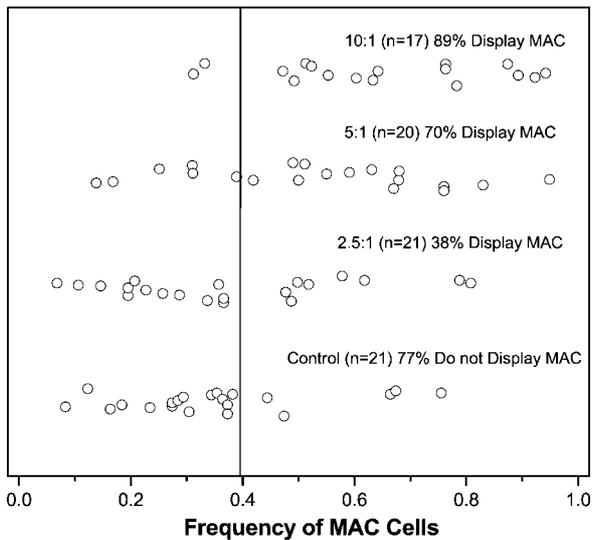


Fig. 2. Frequency of NHBE cells displaying MAC when co-cultured with NCI-H460 tumor cells at ratios of 0 : 1 (control), 2.5 : 1, 5 : 1 and 10 : 1 tumor to normal cell number respectively. Discriminant function was trained to separate normal cells from the 10 : 1 concentration group and the control group and then applied to other concentration groups.

3.3. Dose dependence of MAC expression

Degree of MAC expression in diploid cells varied with the number of malignant cells co-cultured with

NHBE cells for 24 hours. Percentage of NHBE cells expressing MAC was correlated with increased concentration of co-cultured malignant cells. Figure 2 illustrates frequency of cells expressing MAC for different concentration groups. Only NHBE cells from the highest NCI-H460 co-culture concentration group (10 : 1) were used in the training of linear discriminant function against the group of cells, co-cultured with normal cells. The function generated was then applied to the co-culture groups with lower concentration of NCI-H460 (2.5 : 1, 5 : 1); those samples were not used in the function's training and therefore represent an independent test set. Frequency of MAC cells according to the linear discriminant function increased from 38% for the 2.5 : 1 concentration group, to 70% for the 5 : 1, and reached 88% for the 10 : 1 concentration. Statistical analysis using *t*-test for independent samples was applied to compare frequency of cells expressing MAC for different concentration groups versus control group. It revealed significant differences for the 5 : 1 ($p < 0.001$) and 10 : 1 ($p < 0.001$) concentration groups, but not for the 2.5 : 1 concentration ($p > 0.05$).

3.4. Time course of MAC expression

Duration of optimal MAC expression in co-culture experiments was investigated for time periods of 24, 48

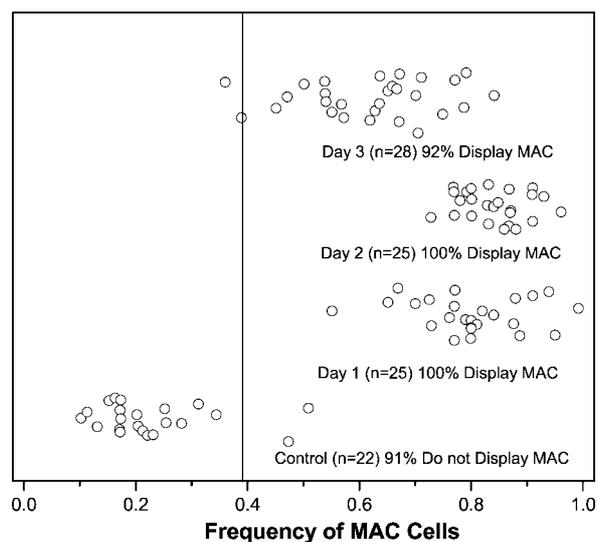


Fig. 3. Time course of MAC expression (X-axis represents frequency of NHBE cells displaying MAC). Discriminant function was generated to separate normal cells from 24-hour time point group and the control group and then applied to 48-hour and 72-hour time point groups.

and 72 hours using a 10 : 1 analysis was performed to separate a group of cells, co-cultured for 24 hours with malignant cells, from the group of controls (NHBE cells co-cultured with normal cells). The resulted function was then applied to groups of samples from the 48 and 72-hour experiments. Frequency of MAC cells was found to be significantly different at every time point when compared with the control group ($p < 0.001$). This difference remained highly significant at the 48-hour time period and kept at that level also at 72-hour co-culture time (Fig. 3). Neither 48-hour group nor 72-hour group was used in training thus representing an independent test set.

3.5. Effect of dose of Fetal Bovine Serum (FBS) on NHBE cell nuclear changes

One of our hypotheses postulated that MAC might simply be a result of any stimulus that moves cells from G_0 stage to G_1 stage and then induces some cells to progress through the cell cycle. To test this hypothesis, 5%, 10% and 20% fetal bovine serum (FBS) was added to the NHBE cell culture medium. We observed significant difference in nuclear features between FBS-stimulated cells and control cells (no FBS added). Stepwise discriminant function analysis between 20% FBS group and control group was performed. Table 2 shows 8 nuclear features that have been picked by the

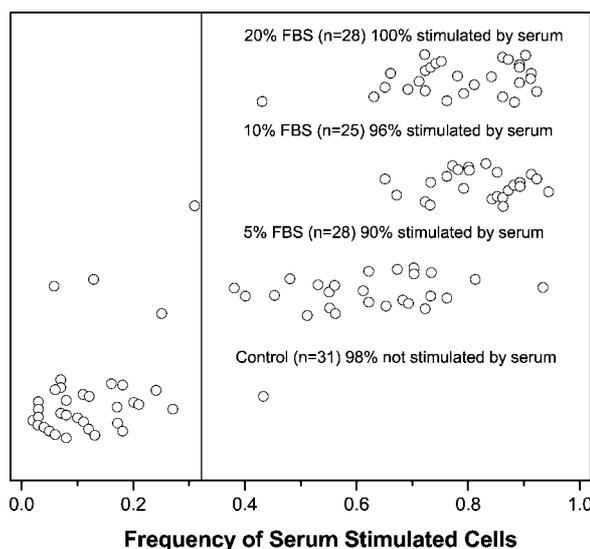


Fig. 4. Frequency of NHBE cells displaying changes stimulated by different concentrations of FBS. Discriminant function was trained to separate NHBE cells from the 20% FBS group and the control group and then applied to other FBS concentration groups.

discriminant function analysis procedure to form a linear function best separating these two groups. This function then was applied to the 5% and 10% FBS groups. As in the case with NCI-H460 co-cultured cells, frequency of NHBE cells displaying changes according to the linear discriminant function was calculated for each sample. This frequency increased from 89% in 5% FBS, to 96% in 10% FBS, and finally to 100% in 20% FBS (Fig. 4). Although serum too affected nuclear features, the key features that could best discriminate serum-stimulated changes were different from those that have been picked in the NCI-H460 co-culture experiment. In order to confirm that the information selected by the discriminant function based on the FBS-stimulated changes is different from that of the MAC expression, we applied the MAC discriminant function to the FBS-stimulated groups (Fig. 5). It is evident that the discriminant function that separates NHBE cells co-cultured with and without malignant cells could not separate NHBE cells stimulated with and without FBS. Therefore, we concluded that malignancy associated changes (MAC) are induced by factor(s) which affect the cells in a significantly different manner from those present in fetal bovine serum.

4. Discussion

In the present study, we have described and characterized the system for studying MAC *in vitro*. A sys-

Table 2

Eight features, selected by the discriminant function analysis to form a linear combination which best discriminated FBS-stimulated and not stimulated NHBE cells

Feature type	Feature name	Feature description
Morphological feature	Sphericity	Measure of object's roundness
Photometric feature	OD_skewness	Measure of asymmetry of optical density distribution
Discrete texture features	MedDNA_area	Percentage of the nuclear area occupied by a medium density chromatin
	HiDNA_area	Percentage of the nuclear area occupied by a high density chromatin
Markovian texture feature	Cluster_prominence	Measure of contrast of chromatin clusters in relation to the background
Non-Markovian texture feature	Range_extreme	Global intensity range estimation
Fractal texture feature	Fractal_dimension	Fractal measure of imaginary 3-dimensional surface created by the optical density function of the nucleus; gives highest values for nuclei with frequent and significant changes of optical density.
	Fractal1_area	Area of imaginary 3-dimensional surface created by the optical density function

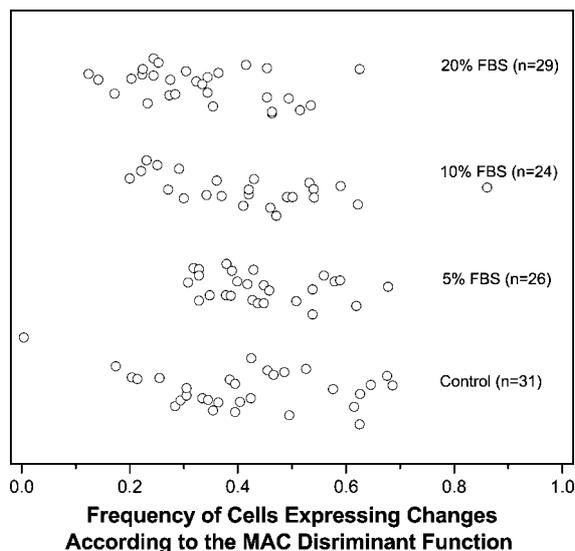


Fig. 5. If the discriminant function from Fig. 4 (FBS-stimulated changes) is replaced by the discriminant function from Fig. 2 (malignancy-associated changes), NHBE cells cannot be separated according to the FBS stimulation grouping (X-axis represents frequency of NHBE cells according to the discriminant function of Fig. 2). Thus MAC features are distinctly different from those associated with FBS stimulation.

tem that utilizes serum-supplemented medium can introduce many unwanted stimulatory and inhibitory factors present in the serum and thus could complicate

the study of MAC. Co-culturing the NHBE (normal cells) with the NCI-H460 (malignant cells) in a serum-free BEGM medium offers a simple model for studying growth factors or other soluble chemical mediators released by the malignant cells that may be responsible for MAC.

Brower et al. [3] reported that NCI-H460 cells can grow in serum-free medium (ACL-3), but we were unsuccessful to passage these cells in serum-free BEGM medium. We therefore implemented a different strategy by growing the NCI-H460 cells first in RPMI medium supplemented with 5% FBS and converting the medium to serum-free BEGM just prior to the co-culture experiments. In this procedure we did not observe any changes in the growth rate or cell morphology. The comparison of the growth rate and morphology of NCI-H460 cells cultured separately in either BEGM or RPMI (5% FBS) medium did not show any significant difference in up to a three-day time period. Thus, we believe that BEGM is a good medium to maintain the NHBE and NCI-H460 cells in co-culture experiments yielding a more defined environment required for *in vitro* studies of MAC.

In our study we found no visible morphological differences between NHBE cells co-cultured with and without malignant cells. However, nuclear features as measured by a high-resolution image cytometer demonstrated significant differences between these

groups. Our data here confirm that an automated, high resolution image cytometry device is sufficiently sensitive to reproducibly measure changes which may not result in morphological manifestations detectable to the human eye.

It has been previously demonstrated that changes in cellular nuclear features as measured by a high-resolution image cytometer could be used as a sensitive tool for the detection of MAC *in vivo* in lung, cervix and other tissues [1,11,14]. MacAulay et al. [15] have found that frequency of MAC expression decreases to the levels seen in normal, control populations after surgical resection of malignant lesions in the lung. Guillaud et al. [11] demonstrated a significant change in size of the cell nuclei as well as sub-visual changes in texture features with different grades of dysplasia, indicating that the intensity of MAC is directly related to the severity of the adjacent lesions. Montag et al. [16], in their study of MAC in colon, demonstrated that frequency of cells expressing MAC was the highest in ostensibly normal cells found in a close proximity of the malignant lesions and decreasing in cells more distant to the cancer boundary. All of the above observations are consistent with the hypothesis postulating that one or more soluble factors are released from malignant cells, which then diffuse to the surrounding tissues resulting in MAC.

The data from this study is consistent with this hypothesis. For example, the increase in MAC expression with the increasing concentration of co-cultured tumor cells (Fig. 2) could be best explained by this hypothesis. In our experiments normal and malignant cells did not physically contact each other, therefore it is reasonable to conclude that malignant cells were affecting normal cells by releasing soluble factor(s) into the medium. It has been reported that lung cancer cells *in vivo* and lung cancer cells *in vitro* produce supra-physiological levels of growth factors and these growth factors act in an autocrine fashion giving the malignant cells a growth advantage [5,12]. It is reasonable to hypothesize that NHBE cells located in the same environment having some receptor(s) for such growth factor(s) would be affected in a paracrine fashion. Further study will be done to confirm the hypothesis that factor(s) are released by malignant cells that induce MAC.

We observed that changes in the nuclear texture features induced in NHBE cells co-cultured with malignant cells were different from those induced by adding serum to the growth medium. These results suggest that MAC are specific to malignant stimulation

rather than just caused by general stimuli that induce cells to go through the cell cycle. Discriminant function that best separates cells with and without MAC could not discriminate between the cells with and without the serum stimulation. Similarly, linear combination of nuclear features discriminating between serum-stimulated and non-stimulated cells could not discriminate between MAC and non-MAC cells. Therefore, we concluded that nuclear changes induced in NHBE cells by malignant cells and FBS are the result of two different stimulatory signal responses.

Due to the technical difficulty of the experiment our study was restricted to a limited sample size; a larger study is needed in order to confirm the results. It should be noted however, that in training of the linear discriminant classifier that separates NCI-H460 co-cultured cells from the control group (MAC expression experiment), we used only cells from the high-concentration group (10 : 1 ratio of tumor to normal cells), leaving other two groups (5 : 1 and 2.5 : 1 concentration) for testing of the classifier (Fig. 2). The fact that the classifier behaved as expected (displaying MAC signal decreasing with tumor cell concentration) gives us confidence in validity of our results. Similar principle (train the classifier using one group of data, test on two other groups) was also used in the experiments studying time course of MAC expression and FBS-stimulated changes.

At this time it is still unclear, what is the biological meaning of the nuclear changes induced by MAC. From our observations, as well as from those of others, it can be speculated that MAC cause chromatin distribution changes in local regions. This may be a result of alterations in DNA supercoiling, which exposes DNA to the transcription machinery. Further studies characterizing MAC are needed to elucidate the biological significance of this phenomenon.

Finally, the MAC-like changes found in these *in vitro* experiments are consistent with those found in experiments *in vivo* that we and others have accumulated over the years. This suggests that our model may be a reasonable reproduction of *in vivo* situation. We believe that with further refinements of this *in vitro* assay, the co-culture system could provide a powerful tool in the investigation of the mechanism(s) of MAC leading to a better understanding of this phenomenon.

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