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Retinoic acid enhances the proliferation of smooth muscle cells

M. M. Peclo and O. Yu. Printseva*

USSR Cardiology Research Center, 3rd Cherepkovskaya 15 A, Moscow 121552 (USSR), 9 December 1985

Summary. Retinoic acid (RA, $10^{-5} - 10^{-7}$ M) is shown to enhance the proliferation of cultured rat aortic smooth muscle cells (SMC). This effect is not connected with a synergistic action of RA together with serum mitogens. Moreover, the expression of L1, a surface antigen specific for modulated SMC entering the cell cycle, is amplified by RA treatment.
Key words. Smooth muscle cells; proliferation; surface antigen; retinoic acid.

The loss of the contractile phenotype and of the proliferation capability of smooth muscle cells (SMC) are considered to be early events in the development of some vascular diseases[1,2]. Screening of drugs able to influence these processes is therefore of considerable importance. On the other hand, derivatives of vitamin A, in particular retinoic acid (RA), have been reported to inhibit the proliferation of normal fibroblasts and of several lines of malignant cells, as well as preventing, in some cases, neoplastic transformation of cells[3,4].

With this in mind, we applied retinoic acid to long-term cultured SMC originally isolated from rat aortic tunica media. As shown previously[5], such cultures retain some characteristics of vascular SMC. In particular, the most remarkable property of the cells appear to be the continual synthesis of the vascular smooth muscle type of actin. The morphology exhibits a series of features that have been considered to be typical of SMC (e.g. typical surface invagination, numerous 'dark bodies'). The results we have obtained show that the proliferation of SMC in vitro is enhanced by RA treatment. Moreover, RA treatment leads to an increase in the expression of L1 antigen which, as shown earlier[6,7], is a specific surface antigen of SMC entering the cell cycle.

Material and methods. Cell cultures. Cell cultures were obtained from the aortic tunica media of adult Wistar rats (250 g b. wt) and from the skin of 17-day-old embryos as described earlier[5]. SMC and skin embryo fibroblasts (SEF) were grown in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS, Flow), 4 mM glutamine and 100 µg/ml kanamycine (Gibco), in an atmosphere containing 5% CO_2. Subcultures of SMC (3rd–30th passages) and of SEF (3rd passage) were used for the two following types of experiments:

1) Cells were seeded in 24-well trays (Falcon) at a density of 2.5×10^4 cm^{-2}. When subconfluency was reached, the growth medium was replaced by serum-free DMEM medium (SF medium) and 24 h later by growth medium containing $10^{-5} - 10^{-7}$ M RA ('all trans', type XX, Sigma) from a stock solution made in absolute ethanol. Wells with growth medium and 0.1% of etha-

Table 1. RA effect on ^{14}CT incorporation in SMC and SEF in the presence of 10% FCS. Results presented in percentage: ^{14}CT incorporation (200.000–500.000 dpm during 1–3 days of experiment) in control wells (without RA treatment) was taken as 100%. The cells were prepared according to protocol 1. The level of ^{14}CT non-specific absorption was about 100 dpm. Each value is the mean of three separate determinations

RA treatment	Duration of treatment, days 1	2	3
SMC			
10^{-5}M	157 ± 13^b	166 ± 6^a	565 ± 25^a
10^{-6}M	159 ± 2^a	146 ± 9^b	181 ± 100 (ns)
10^{-7}M	129 ± 6^c	101 ± 10 (ns)	67 ± 45 (ns)
SEF			
10^{-5}M	74 ± 3^b	58 ± 1^a	56 ± 3^a
10^{-6}M	84 ± 5^a	61 ± 4^a	61 ± 3^a
10^{-7}M	86 ± 6^c	72 ± 4^a	68 ± 3^a

^{a,b,c} Significant differences according to Student's t-test with $p < 0.001$, 0.01, 0.05 respectively; (ns) not significant.

Table 2. RA effect on ^{14}CT incorporation in SMC and SF medium (percentage). ^{14}CT incorporation (50.000–75.000 dpm during 1-4 days of experiment) in control wells, without RA treatment, was taken as 100%. The cells were prepared using protocol II. The level of ^{14}CT non-specific absorption was about 100 dpm. Each value is the mean of three separate determinations. All differences were significant using Student's t-test with $p < 0.001$.

RA treatment	Duration of treatment, days 1	2	3	4
10^{-5}M	116 ± 1	126 ± 2	219 ± 1	179 ± 3

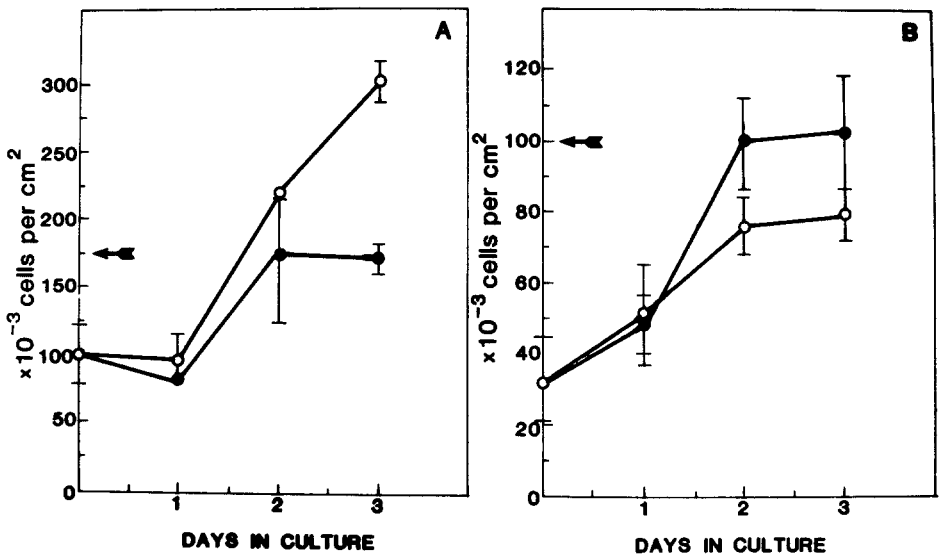

Figure 1. Growth curves of SMC (A) and SEF (B) with (open circles) and without 10^{-5} M RA (closed circles). The cells were prepared as described in protocol I. The final density of cells in controls is marked by arrows. Each value is the average of three separate determinations.

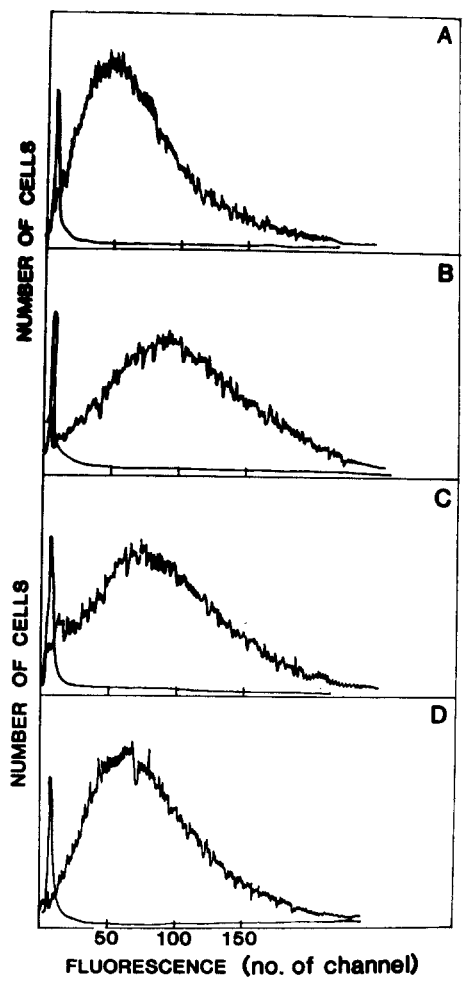

Figure 2. Results of flow cytofluorometric analysis of cells in the presence of 10% FCS. The cells were prepared as described in protocol I. The smooth curves represent non-specific fluorescence (second antibody alone). *A* control; *B, C, D* 10^{-5}, 10^{-6}, 10^{-7} M RA, respectively.

nol (corresponding to the maximal concentration of solvent used in RA containing wells) served as controls.

2) Cells were seeded in 60 mm tissue culture dishes (Nunc) at a density of 3×10^4 cm^{-2} in growth medium. Upon attaining subconfluency, cells were washed twice with Hank's balanced salt solution to remove the residual serum, then kept in SF medium containing 10^{-5} RA. Control wells with SF medium received 0.1% ethanol.

^{14}C-thymidine incorporation. ^{14}C-thymidine incorporation was studied during the first 4 days of RA treatment: the cells were supplemented with ^{14}C-thymidine (50 µCi/mmole) up to a final concentration of 1 µCi/ml for 17 h. Cells were then placed on GF/C filters (Whatman); the filters were dried and the sample radioactivity measured in a liquid scintillation counter (LKB Rack-beta 1215).

Flow cytofluorometry. Cells were prepared for flow cytofluorometry studies as described in protocols I and II; after detachment by trypsin-EDTA treatment, cells were stained by an indirect immunofluorescence method using successively the L1 antibody[6] and FITC-conjugated rabbit anti-mouse Ig. Cells stained by the second antibody alone were used as control samples. Analysis was carried out on a cell sorter (FACS II). The results are presented as histograms. To determine the mean intensity of the fluorescence per one cell computer analysis was performed in accordance with a program which permits the expression of the fluorescence intensity per cell in relative units as:

$$\frac{\text{channel number} \times \text{cell number in individual channel}}{\text{number of cells}}$$

Results and discussion. Experiments carried out using subcultures of SMC either at 3rd or 21st–30th passages gave similar results under all experimental conditions.

In the presence of 10% FCS, RA ($10^{-5} - 10^{-7}$ M) increased the level of ^{14}CT incorporation in a dose-dependent manner (table 1). The comparison of growth curves shows that the number of cells on the 3rd day of culture increased nearly two-fold in response to RA (fig. 1). At the same time in control cultures, the cells have reached the final density characteristic of the cell type. Therefore, RA not only enhances the proliferation of SMC but also delays the exit of cells from the proliferative cycle in dense monolayers, increasing the saturation density of the culture. RA, in the concentration range of $10^{-5} - 10^{-7}$ M, has been reported to inhibit the proliferation of fibroblasts of different

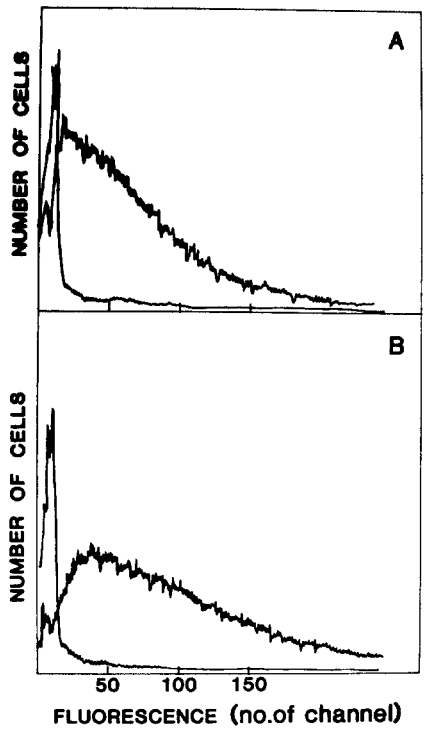

Figure 3. Flow cytofluorometric analysis of cells maintained in SF medium. Protocol II. The smooth curves respresent non-specific fluorescence (second antibody alone). *A* control; *B* 10^{-5} M RA.

origins[8–10]. These data are in complete agreement with the results we have obtained in control experiments with SEF (fig. 1, table 1). Thus, the observed stimulating action of RA is specific for SMC.

Several investigators have observed a similar effect of RA on certain eucaryotic cells. They reported that RA can enhance the proliferation of tracheal epithelium, Swiss 3T3 and rat kidney cells[11–13]. However, this stimulation of growth by RA was shown either in the presence of fetal serum and mitogens or in serum-free medium supplemented with various growth factors. These data suggest that the effect of RA on cell growth results from a synergistic action together with exogenous mitogens[3]. This coincides with observations that the binding of epidermal growth factor (EGF) with specific surface receptors is increased when cells are treated by RA[13].

It is known that the growth of SMC is stimulated by a platelet-derived growth factor (PDGF) contained in whole blood serum[14]. Therefore we studied the influence of RA on the proliferation of SMC in serum-free medium to test for a possible synergistic action of RA with PDGF or other serum mitogens. In this experiment, RA was used at 10^{-5} M, a concentration which leads to the maximal stimulation of the proliferation of SMC in the presence of FCS. A stimulating effect of RA on ^{14}CT incorporation was also observed in serum-free medium (table 2) suggesting that RA alone can be a stimulating factor.

Recently, we have selected a monoclonal antibody (L1) which reacts only with the surface of long-term cultivated, modulated SMC. As we have shown earlier, there is a correlation between the rate of expression of L1 and the state of cell proliferation[6,7]. The results obtained in the present study agree with this observation. The expression of L1 antigen was stimulated by RA in a dose-dependent manner. Histograms of cell distribution with respect to L1 fluorescence intensity are presented in figure 2. After RA action intensity curves are shifted to bigger channel numbers. The maximal shift was observed at RA concentration 10^{-5} M (fig. 2B). Computer analysis additionally revealed that the fluorescence per one cell increased by 1.5-fold after 10^{-5} M RA treatment in serum-free medium (fig. 3).

The function of L1 is still unknown. A polypeptide of 95–105 kD was found to be precipitated from SMC lysates by L1 antibody (unpublished data).

At the present time, little is known about the mechanisms of action of RA, though a few hypotheses have been presented. In particular, it has been suggested that phosphorylated derivatives of retinoids can serve as donors of mannose for membrane glycosyl-transferase reactions[15]. Via this intermediate, certain glycosylation reactions could be modified, resulting in altered cell surface molecules. The change in L1 expression on the surface of SMC after RA treatment may be due to this mechanism. The results obtained here show that RA treatment produces a specific enhancement of the SMC proliferation which correlates with an increased expression of the L1 antigen. This model may thus be a potential tool for the study of L1 antigen function(s).

* To whom correspondence should be addressed.

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