BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 154–158 (1997)
ARTICLE NO. RC977218

A Novel Aortic Smooth Muscle Cell Line Obtained from p53 Knock Out Mice Expresses Several Differentiation Characteristics

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Received July 25, 1997

Here we report that we could obtain a highly differentiated smooth muscle cell line by screening the expression of a-smooth muscle actin from p53 knock out mice aorta. This cell revealed extended bipolar shape and expressed h-caldesmon and calponin as well as a-smooth muscle actin as protein markers of differentiated smooth muscle. Further intracellular calcium increase was induced by application of noradrenaline in a dose dependent manner and calcium oscillation was also observed in a higher dose (100μM). Appropriate application of 5-azacytidine enhanced these tendencies and induced slow contraction by endothelin-1 and phenylephrine.

Vascular smooth muscle cells in culture lose contractility (1,2), accompanied by changes in the expression of smooth muscle-specific protein markers (3-7). Establishment of the *in vitro* differentiation-inducible system concerning the cells is significant for the physiological and pathological study. Tumor-suppressor gene, p53 is an interesting gene in some relation to cell growth, differentiation, and apoptosis (8-11). Several long-term cultures have been prepared from several tissues of p53 knock out mice (12,13). To establish a differentiated vascular smooth muscle cell line, laborious effort has been done by many laboratories for long time, but it remains unsolved yet. Then we have started various trials to obtain differentiated cell line or the cell line with such a tendency. Thus p53-deficient mouse might be a good material for the study of cell growth and differentiation. We have started to prepare a cloned cultured cell of aortic smooth muscle from p53 knock out mice.

MATERIALS AND METHODS

Establishment of new smooth muscle cell line from p53 knock out mice aorta. The thoracic aorta of three months old p53 deficient (−/−) mice (14) was dissected and connective tissue as well as fat were removed. After endothelial layer was rubbed out, smooth muscle layer was cut into small pieces and incubated in the medium containing 20U/ml elastase (Sigma) and 1mg/ml collagenase (Sigma) at 37°C for 2 hours. The tissue suspension was filtered with a nylon filter, centrifuged at low speed for short time, and the pellet was resuspended in growth medium (DMEM supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). The cells were incubated in humidified 5 % CO_2-95 % air atmosphere at 37°C. The cell was cloned by plating 10 cells per one well in a 96-well plate and incubated in the growth medium. Then each colony was transferred to a 24-well plate and cultured. Individual cells were further subcultured and the cells expressing a-smooth muscle actin were selected by immunoblotting with the antibody. These cells were subcultured, multiplied, and cloned again. Finally the cells expressing h-caldesmon induced by 5-azacytidine were selected.

Immunoblotting. Aorta from SV129 mice was cleaned by exclusion of fats and connective tissue. P53LMACO1 cells were seeded into 100 mm dish and cultured in growth media. After 24 hours the media were replaced by growth medium containing 5-azacytidine and the cells were cultured for 3 days. Or the cells without 5-azacytidine were cultured several days till confluent state. Then tissues and collected cells were homogenized with the sample buffer for SDS-PAGE. Soluble fractions were separated by centrifugation from tissues and cells debris. Ten mg of proteins from each extract were applied to each lane for SDS-PAGE. After transferred to nitrocellulose membranes (S & S) samples were reacted with first antibodies of a-smooth muscle actin (monoclonal, Sigma), calponin (monoclonal, Sigma), and caldesmon (polyclonal, produced in our laboratory). Then alkali phosphatase-conjugated second antibodies were reacted. The membranes were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

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0006-291X/97 $25.00

Intracellular calcium measurement. Intracellular Ca^{2+} was measured by an inverted fluorescence microscopy (Olympus IMT-2) with an image processor (Hamamatsu photonics, Argus 50). The ratio at 340 nm to 360 nm of fluorescent images of a fura PE-3 (IEFLABS) loaded specimen gave the relative magnitude of intracellular Ca^{2+} changes (15). Just before the experiment the cells were washed with BSS and incubated in BSS containing 5mM fura PE-3 (16) for more than 30 min. at 30°C.

Contraction of P53LMACO1 cells. Cells were seeded on the flex II amino plate (Iwaki glass) coated with collagen type I-C (Iwaki glass) at a density of 2×10^4 cells per cm^2 in growth medium. After 12 hours the cultured media were replaced with growth medium containing 5-azacytidine and incubated for more than 6 days. The cells without chemical were used as control. Control and chemical treated cells were set up under a phase contrast microscopy equipped with video recording system and incubated with each medium at 30°C. Growth media were replaced with the solution containing 1 μM phenylephrine and 1 nM endotheline-1 after washing by BSS twice for 5 min.

RESULTS AND DISCUSSION

First we isolated smooth muscle cells from aorta of p53 deficient (−/−) mice produced in place of the p53 gene to the lac gene (14) and cultured these cells according to the standard procedure. After several passages from primary culture the cells were cloned by limiting dilution of cells. Proliferation of the cell was very well and several clones expressing a-smooth muscle actin were selected by immunoblotting of extracts from whole homogenates of the cells. Cell feature of this stage was deformed and ununified flattened shape in the confluent stage (Fig. 1D), being different from a typical cell shape of smooth muscle cell, bipolar shape. This procedure described above seemed to be disadvantageous for preparation of differentiated cell line, because we might select only the cell clone proliferating rapidly. So that a direct cloning method was utilized to the primary culture as described in Methods. Thus several clones expressing a-smooth muscle actin and revealing spindle-shaped feature, were selected as shown in Fig. 1A, and in confluent state the cell became elongated, arranging in parallel (Fig. 1C), which were considered to reflect the characters of original adult cells used for the cells' preparation. These clones expressed h-caldesmon as a marker of differentiated smooth muscle cell (3), though its amount was small as compared with expression of 1-caldesmon. Next, several chemical agents were tested to get effective one which induced an increase of h- to l-caldesmon ratio as an index of cell differentiation. As results of many trials 5-azacytidine was found to be a best agent for this purpose. One out of several clones tested was most effective from the point that h-caldesmon was highly induced by 5-azacytidine treatment. The shape of the cell looked more symmetrically bipolar (Fig. 1B) than that of un-

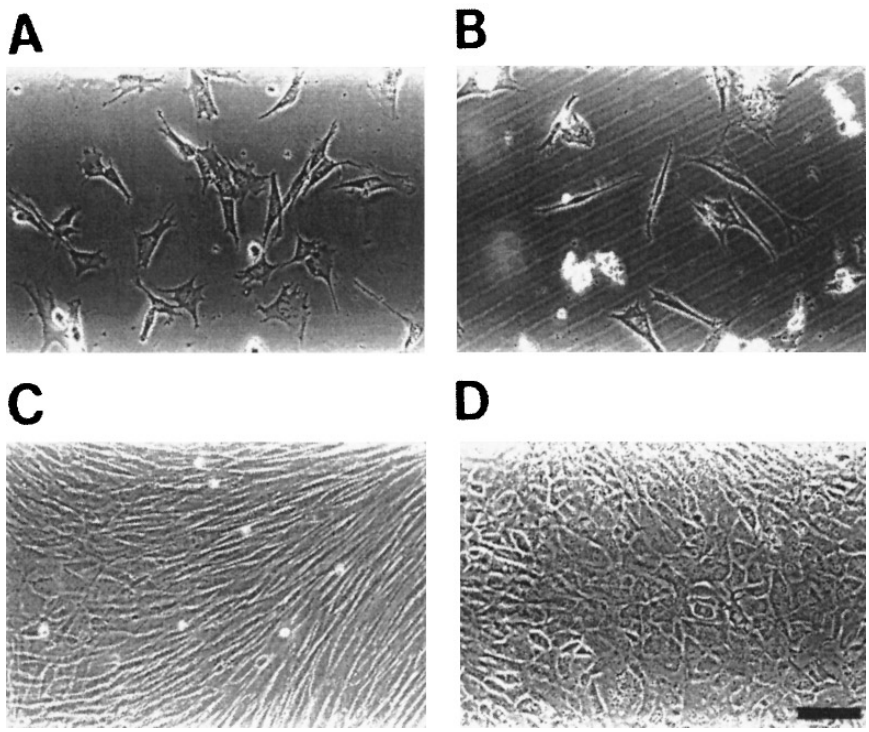

FIG. 1. Phase contrast photomicrographs of cultured P53LMACO1 cells with (B) or without (A and C) 5-azacytidine, and another clonal cultured aortic smooth muscle cell (D, clone No. 4). A, log phase stage (2 day); B, 3 day's culture; C, confluent stage (6 day); D, confluent stage (6 day). Scale bar is 100μm.

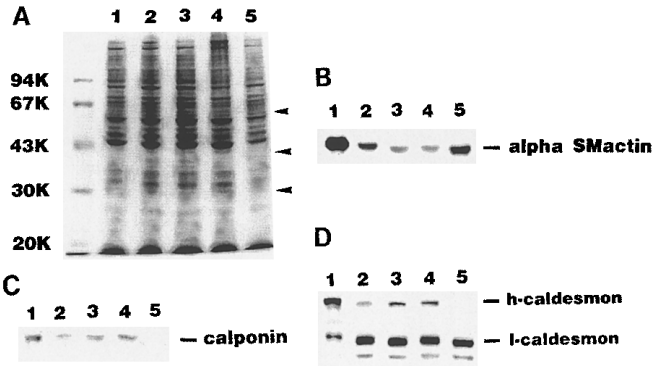

FIG. 2. Immunoblotting analysis of expression of contractile proteins in P53LMACO1 cells and adult aortic smooth muscle. A, protein staining of Western blots of extracts. Arrowheads show the cut positions for immunostaining; B, immunostaining with anti α smooth muscle actin; C, with anti caldesmon; D, with anti calponin. Lane 1, extract from SV 129 mouse adult aorta; lane 2, from log phase cells; lane 3, from 3 days' cells treated with 30μM azacytidine; lane 4, from 3 days' cell treated with 100μM azacytidine; lane 5, from confluent stage cells.

treated cell, asymmetrical spindle shape (Fig. 1A). The growth of the treated cell was suppressed by 5-azacytidine in a dose dependent manner. This clone was established as a cell line by many passages for over six months and it was named as P53LMACO1.

Expressions of contractile proteins in both log phase and confluent state of the cell line with or without 5-azacytidine were examined by immuno-blotting. Alpha-smooth muscle actin was expressed in all materials: confluent state, strong; 5-azacytidine treatment (10 to 100μM), weak in log phase compared with no treatment (Fig. 2B). H-caldesmon and calponin appeared in log phase though with small amount without the treatment (Fig. 2C and D lane 2), while in confluent state both proteins disappeared (Fig. 2C and D lane 5). Both proteins were relatively strongly expressed by 5-azacytidine treatment in concentration-dependence (Fig. 2C and D lane 3 and 4).

To check the grade of differentiation in the cultured vascular smooth muscle cells, it might be the best way to determine the expression of some receptors, particularly a-adrenergic receptor in the surface membrane. For this purpose the change in intracellular concentrations of Ca^{2+} as a response to noradrenaline was examined by measurement of ratio of 340 to 360 nm fluorescent intensities (15) using of fura PE-3 (16). P53LMACO1 cells showed marked increase of intracellular Ca^{2+} by noradrenaline more than 10^{-6}M. Oscillatory tendency was also observed in increase of noradrenaline concentrations (Fig. 3A). This tendency was remarkably observed in the 5-azacytidine treated cells dose-dependently (Fig. 3B to D). Direct type of a-receptor agonist, phenylephrine showed the same effect as noradrenaline in almost the same dose (data was not shown). Relatively selective a-receptor antagonist, phenoxybenzamine blocked Ca^{2+} response to noradrenaline in a dose dependent manner (not shown).

Muscle contraction is most basic function of vascular smooth muscle, but the case reporting the contraction of vascular smooth muscle in culture by agonist has not been found yet in spite of abundant reports dealing with differentiation of cultured cells. When P53LMACO1 was tested by phenylepherine and endotheline-1, approximately 5 %of the cell could contract by stimulation of both agents (data not shown). On the other hand, approximately 30 %of the 5-azacytidine treated cell induced contraction with both agents (Fig. 4A to D). For clear demonstration these figures showed the results after 15 min. of stimulation, but practical contraction was observed from about 1 min. later. Contracted change of the cell was not observed under the state of BSS (basal salt solution) in both cells with or without 5-azacytidine treatment.

While P53-deficient cell would be, generally speaking, predicted to become undifferentiated from its gene character, tumor suppressing gene (8); lack of inhibiting the undifferentiating state, oncogenesis had been expected to become more undifferentiating, more growing, and immortal (12,13). We were surprising to get our remarkable results in which p53-deficient aortic smooth muscle cell showed good cell proliferation and finally immortalization as our expectation, but rather unexpected characters were observed; several noteworthy differentiated characters. Although the mechanism of differentiation by p53 knock out is not solved yet, we are now searching the strategy to approach this problem.

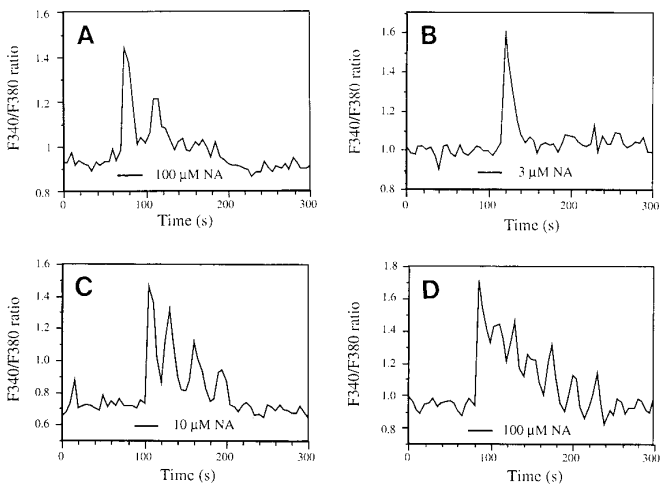

FIG. 3. Transient intracellular calcium increase of P53LMCO1 cells to noradrenaline application. A, cell response to 10^{-4}M noradrenaline without 5-azacytidine; B-D, cell response to various concentrations of noradrenaline with 5-azacytidine. Notice Ca^{2+} oscillation in C and especially D. Inserted bars show applied period of various concentrations of noradrenaline.

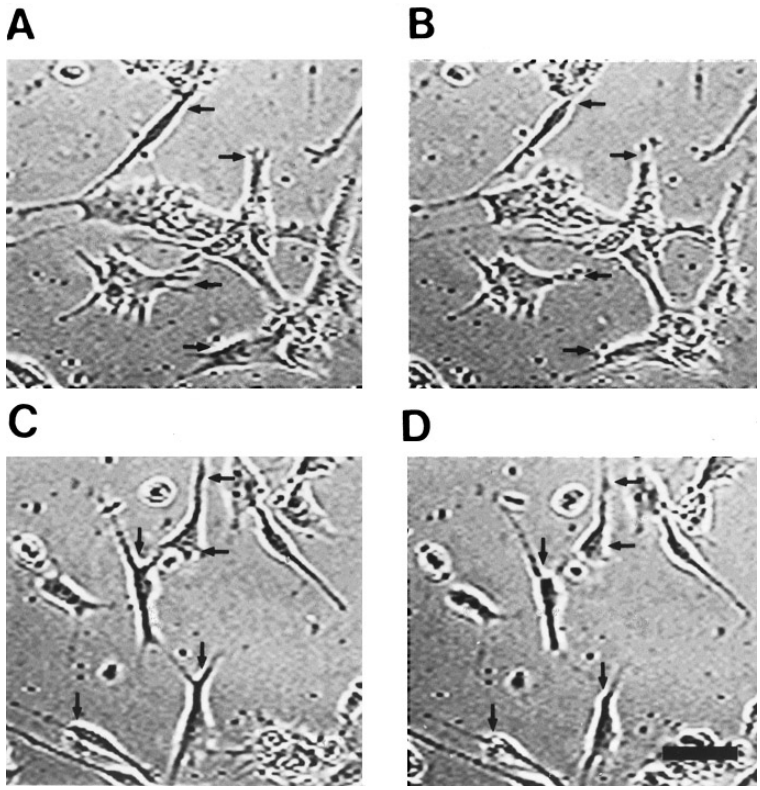

FIG. 4. Phase contrast photomicrographs of the contracted process of P53LMACO1 cells induced by phenylephrine and endothelin-1. Cells were treated with 100μM 5-azacytidine for 6 days. Cells before (A) and cells after application of 1 μM phenylephrine for 15 min. (B) Cells before (C) and cells after application of 1 nM endothelin-1 for 15 min. (D) Arrowheads show marked contracted cells before and after 15 min. stimulation of agents. Scale bar is 50 μm.

Alpha smooth muscle actin and calponin had been considered to be protein markers as differentiated smooth muscle previously, but recently their specificity for adult state has been suspicious, because these proteins have expressed in the embryonic state or non muscle cells (17-20), while h-caldesmon seems to be very specific for adult smooth muscle (3). There has been no success to get clear expression of h-caldesmon in the lined vascular smooth muscle cell and even in primary cultured cells, for example, though recently it was reported by two groups to have gotten relatively differentiated smooth muscle cells using retinoic acid treated P19 cell (21,22).

Our success in demonstrating noradrenaline response and particularly Ca^{2+} oscillation in P53LMACO1 cell is a first report in vascular cultured smooth muscle cells. Since expression of a-adrenergic receptor was reported to be a post-natal event (23), its appearance might mean strongly advanced differentiation.

5-azacytidine treatment was known to convert the mouse lined cell, C3H10T1/2 into differentiated chondrocyte and skeletal muscle cell by demethylation of DNA; incorporation of the chemical in place of methylated site of DNA (24,25). Although the mechanism of enhancement on differentiation with 5-azacytidine in P53LMACO1 cell is not understood yet, the enhancement of differentiation seems to be reasonable tendency from the results in C3H10T1/2 cell. Such a effect of 5-azacytidine could not be generalized in undifferentiated smooth muscle cell, because it had no effect to primary cultured smooth muscle cell from usual adult rat aorta (data not shown).

ACKNOWLEDGMENTS

Late Dr. Seiji Nakamura had first planned this work and without his prediction this project would not be accomplished. We thank Prof. Masamitsu Iino Dr. Yuzuru Matsuda for supporting this work. This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Science and Sports of Japan.

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