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Report on the Differentiation of Vascular Wall Smooth Muscle Cells with the Aid of Immunofluorescence* **

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Summary. The early differentiation of smooth muscle cells from fibroblasts in tissue cultures of vascular wall explantates has been for the most unsuccessful due to morphological criteria. With fluorescent-marked rabbit antiserum against chicken gizzard smooth-muscle actomyosin apple-green positive fluorescence in round cells is already detectable after one day. It is these myocytoblasts which differentiate into myocytes in approximately 6 days after forming uni- and bipolar processes. This method allows the distinct separation of vascular wall smooth muscle cells from fibroblasts, a differentiation which is of importance for the analysis of metabolic processes in vascular changes due to arteriosclerosis.

Key words: Vascular Wall Smooth Muscle Cells — Immunofluorescence — Chick — Tissue Culture.

Zusammenfassung. Die Frühdifferenzierung glatter Muskelzellen von Fibroblasten im Gefäßwandexplantat von Gewebekulturen war bislang auf Grund morphologischer Kriterien nicht oder nur bedingt möglich. Mit einem fluorescenzmarkierten Kaninchen-Antiserum gegen Aktomyosin aus Hühnermuskelmagen ist bereits 1 Tag nach Kulturansatz eine apfelgrüne positive Fluorescenz in runden Zellen nachweisbar. Es sind dies Myocytoblasten, die sich nach Bildung uni- und bipolarer Fortsätze in aller Regel nach 6 Tagen zum Myocyten differenzieren.

Diese Methode ermöglicht die Trennung definierter glatter Gefäßwandmuskelzellen von Fibroblasten, eine Unterscheidung, welche für die Analyse von Stoffwechselvorgängen in arteriosklerotischen Gefäßveränderungen wichtig ist.

An exact description of the formal morphogenesis of vascular wall smooth muscle cells has not been successful despite numerous attempts. The important role of the smooth muscle cell in the formation of the so-called atheroma requires some explanation as to its origin. The discussion revolves around the thought that vascular wall muscle cells are derivatives of fibroblasts or mesenchyme cells.

Under certain physiological and pathological conditions the vascular smooth muscle cells could 'undifferentiate' and regress to active muscle cells losing contractile function and acquiring synthesis and phagocytic properties (Weiss, 1968). Collagen precursors, elastin and matrix are supposedly synthesized (Karrer *et al.*, 1960; Knieriem, 1970; Takebayashi *et al.*, 1972). The organization of myofilaments has been partially elucidated through the use of the electron microscope, however many questions remain unanswered (Gansler, 1956, 1960, 1961), especially the localisation and distribution of myosin in the cytoplasma and the actual con-

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traction process. Panner and Honig (1967) as well as Lowy and Small (1970) describe a sliding mechanism while Fay and Delise (1973) have recently demonstrated herniations of the cell membrane with reorientation of the largely longitudinally positioned myofilaments in the noncontractile state to mostly indescrimminantly placed formations during contraction. Chemical methods have proven the presence of actin and myosin in a molar ratio of 1:4 (Needham and Williams, 1963). Knieriem and coworkers (1966, 1967, 1970), Kao and coworkers (1968) and Becker and Nachman (1973) could clearly differentiate smooth muscle cells with the aid of fluorescent-marked antiserum.

The purpose of this study is: 1. With the aid of fluorescentmarked antiactomyosin serum to recognize early vascular wall smooth muscle cells in a mixed cell population from a tissue culture and be able to follow their growth and differentiation.

2. To establish whether the vascular wall smooth muscle cells arise from fibroblasts.

3. To find an exact and simple method of differentiation between vascular wall smooth muscle cells and fibroblasts with respect to future metabolic experiments with cloned vascular wall smooth muscle cells.

Materials and Methods

Incubated chick embryos 11 to 18 days old, Nichols race, were opened under sterile conditions and the vessels (descending aorta, pulmonary artery, left and right brachiocephalic arteries and severed from the heart in approximately 9 mm lengths. These vascular entities were stripped externally of connective tissue, the adventitia removed as best as possible, and then slit open longitudinally. Two mm square-cut explantates were placed on coverslips in a small tissue culture petri dish (Greiner) with TC 199 (Difco) + 10% fetal calf serum and then in an incubator at 37° C, 95% O_2 and 5% CO_2 gas flow for cultivation. The cellular growth was followed daily, medium change every end to 3d day. Photography: Leitz Diavert, phase contrast. Film: Ilford Pan F, 18 DIN (England). Following fixation with ice-cold methanol the cells were exposed to anti ch GAM[1] (rabbit antiserum against chicken gizzard smooth muscle actomyosin) according to the method of Coons for 30 min in a humidified chamber, washed for 30 min with CMF-PSB puffer and covered with Glycin-puffer (pH 6.8). The preparations were examined under a Leitz Ortholux Fluorescence microscope using the filter combinations BG 480 and K 510, Hg 200 W lamp and photographed with high-speed Ektachrome positive film for documentation.

Results

The initial cell buds are already recognizable after 20 to 24 hours of incubation. Forty-two hours later the cells are noticably more populous and stretched in shape (Fig. 1). After 10 days the cell culture is quite thick, cell bordering on cell. At 21 days the periphery loosens, the cells demonstrate broad cytoplasma formations and fat vacuoles (Fig. 2). Smooth muscle cells and fibroblasts occur together in the culture without being separately discernable as such. As early as the sixth day cells with thin uni- and pipolar cytoplasmatic processes can be seen (Fig. 3).

Following treatment with antiactomyosin these cells demonstrate a prominent apple-green cytoplasmatic fluorescence (Fig. 4a and 4b). Positive cytoplasmatic fluorescence is already present on the first day inside small, round cells with large nuclei at the rim of the explantate (Fig. 4c). After the 14th day a broad cytoplasma

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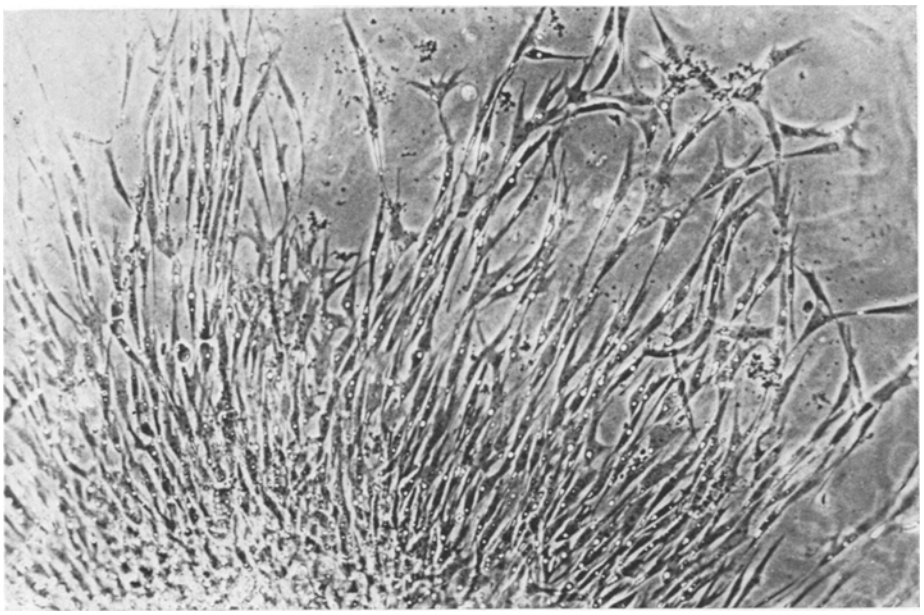

Fig. 1. Cell growth after 42 hours: cells lie quite close and are for the most part longitudinally stretched. Phase contrast, mag. × 100

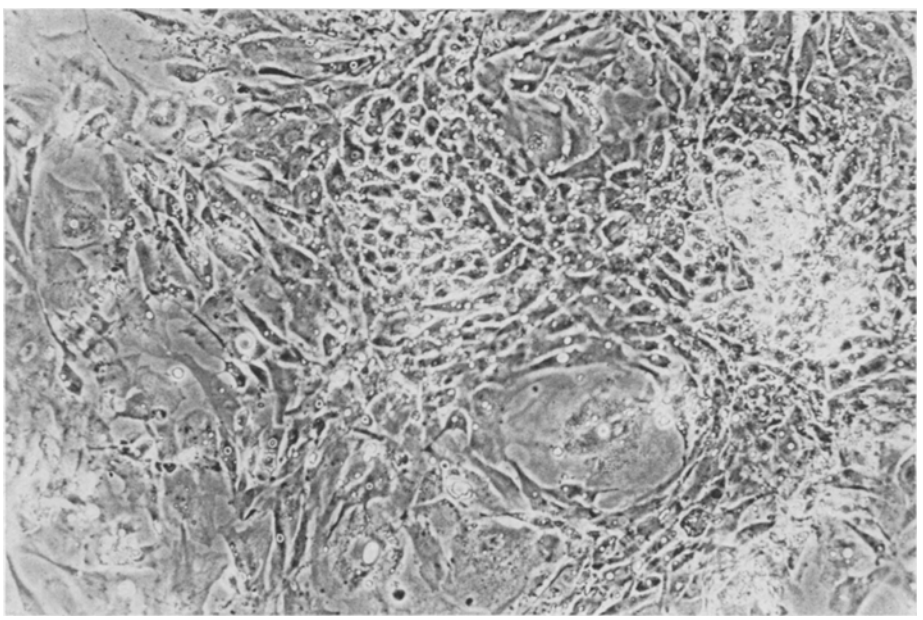

Fig. 2. Cell growth after 21 days with marked loosening in the periphery. Cells demonstrate broad cytoplasmatic formations. Phase contrast, mag. × 100

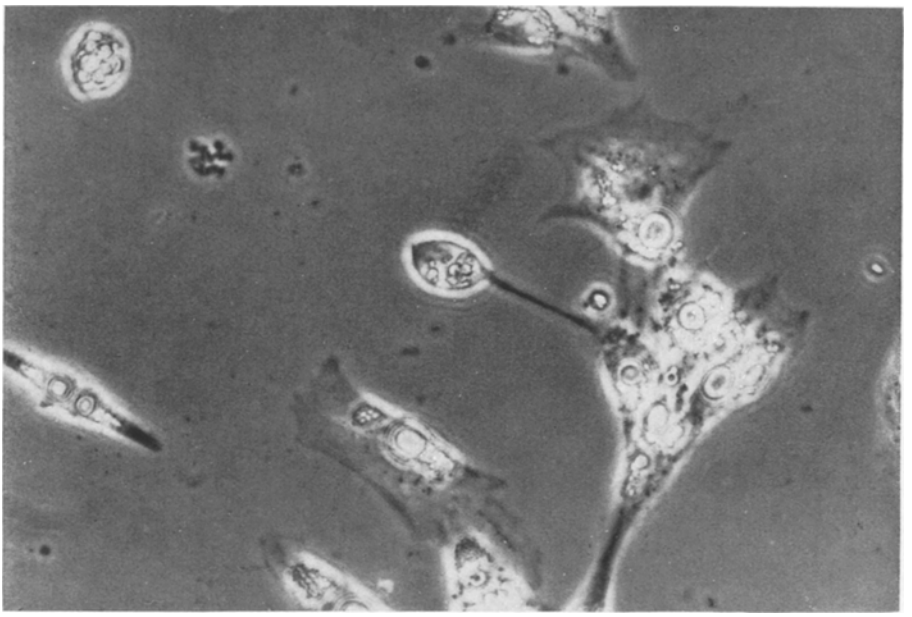

Fig. 3. Cell growth after 6 days: cells with uni- and bipolar cytoplasma processes. Phase contrast, mag. × 300

Table 1

Culture date	Age of Embryo (days)	Culture medium	Fixation substance	Number of days elapsed before positive fluorescence
I 5/8	12–13	EAGLE's +10% Horse-S.	Methanol	13, 16, 17
II 5/12	16–17	EAGLE's +10% Horse-S.	Methanol	10, 12, 13, 16
III 5/29	13–14	TC 199 +10% FKS	Methanol	8, 9, 10, 14, 15
IV 6/26	16–18	TC 199 +10% FKS	Methanol	6, 8, 9, 10, 13, 14, 15, 16, 17, 20, 23, 28
V 7/17	11–12	TC 199 +10% FKS	Methanol	2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15
VI 9/19	13	TC 199 +10% Horse-S.	Methanol	1, 2, 5, 7, 8, 12, 14, 15, 20, 23

ring forms around the cell nucleus in which very fine actomyosin filaments are discernable in fluorescent light (Fig. 4d). During this phase of growth cells which present at right angles to the main growth direction appear. A tabulation of the positive cytoplasmatic fluorescence is shown in Table 1.

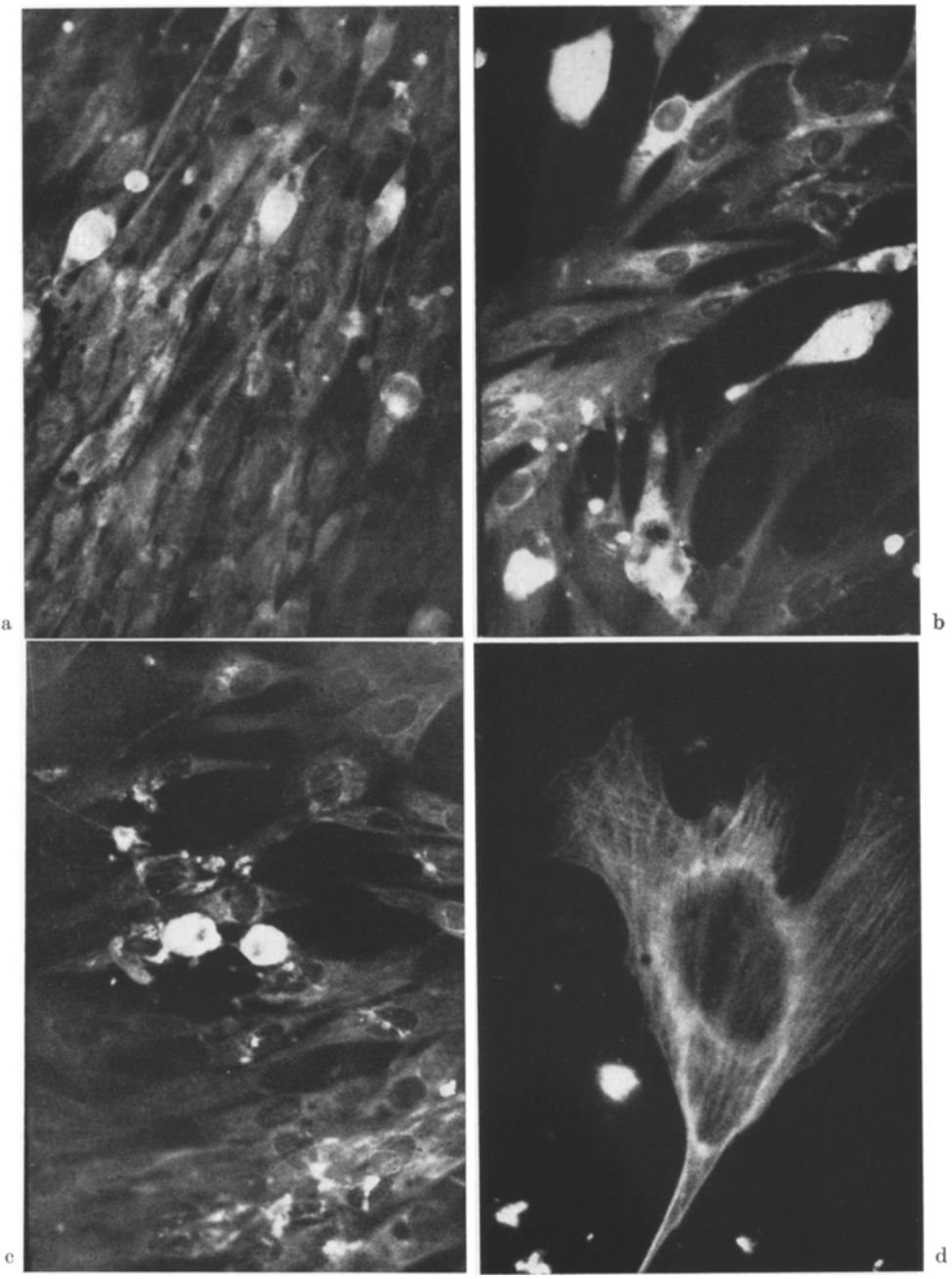

Fig. 4. a Positive, apple-green cytoplasmatic fluorescence in cells with bipolar processes after 6 days. Fluorescent picture $\times 400$. b Positive apple-green cytoplasmatic fluorescence after 9 days. Fluorescent picture $\times 400$. c Positive, apple-green cytoplasmatic fluorescence after 1 day; two small, round cells with large nuclei. Fluorescent picture $\times 400$. d Positive apple-green cytoplasmatic fluorescence of actomyosin filaments after 14 days. Fluorescent picture $\times 950$

Discussion

Our experiments show that contrary to the reports of Kasai and Pollak (1964) and Pollak and Adachi (1968), the vascular wall smooth muscle cells and their precursors are already present on the first or second day after culturing the vessel explantate. Initially these cells are smaller than fibroblasts and round in form. The nucleus is relatively large, the cytoplasma demonstrates a marked, apple-green homogeneous fluorescence. These characteristically formed cells are able to divide. We assume that these are myocytoblasts which are present in the vascular wall as differentiated cells and stem from embryonic mesenchyme cells. These cells often lie together in small aggregates. After 3 to 4 days they form processes in one direction and become somewhat spindle-shaped. After this form change no further nuclear divisions occur. A few days later they assume a roughly triangular form with thin cytoplasmatic fingers. The cytoplasma often demonstrates a homogeneous, heavy fluorescence together with apple-green longitudinal structures which we believe to be actomyosin filaments. On the 14th day well-differentiated myocytes are present.

The phenomena of the right-angle crossing of fibroblasts and smooth muscle cells as observed by Pollak (1964) and by Pollak and Kasai (1964) could be confirmed, however, whether these cells crossing at right angles are myocytes cannot be definitely stated.

As has been mentioned above, small, round cells with specific fluorescence are already seen on the first and second day (myocytoblasts). They most likely stem from the embryonic mesenchyme which largely develops from the mesodermal flank plates—the splanchnopleura—(Goerttler, 1963). These pluripotent mesenchyme cells differentiate into various cells during the embryonic development, i.e. fibroblasts, reticulum cells, osteoblasts, chondroblasts, fat cells, muscle cells, and blood vessels (Hueck, 1920; Thoma, 1920; Starck, 1955; Leonhardt, 1971). In a noteworthy study, Hughes (1943) demonstrated that from the 4th day on in the embryonic development of the chick aorta, numerous mesenchyme cell complexes form which lie immediately around the endothelium and differentiate first to myocytoblasts and then myocytes after the 9th to 11th day.

Our experiments have also shown that the pluripotent mesenchyme cells differentiate early into myocytoblasts and these again divide. After the 7th day they develop into myocytes. A differentiation from mesenchyme cells to fibroblasts and then myocytes could not be observed.

With this relatively simple method of immunofluorescence it is possible to recognize early vascular wall smooth muscle cells. The further investigations with cloned smooth muscle cells from a defined cell strain attempt to describe the metabolic magnitudes and changes under defined experimental culture conditions.

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