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A Simple Method for Differentiating Vascular Smooth Muscle Cells and Fibroblasts in Tissue Culture*

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Summary. The morphologic differentiation of vascular smooth muscle cells and fibroblasts in tissue culture is difficult if not impossible. By direct immunofluorescence, it is possible to distinguish between vascular smooth muscle cells and fibroblasts after 6 to 10 days in tissue culture. Microfilaments appear from the 6th to the 10th day. After an incubation period of 30 minutes with antibody against smooth muscle actomyosin at room temperature, microfilaments are demonstrable in smooth muscle cells. In contrast, fibroblasts, if incubated for the same period, show strong nuclear fluorescence and a primary fluorescence of the cytoplasm, but filaments are *not* visible. If fibroblasts are incubated with antiactomyosin for *one hour* at 37° C, however, microfilaments are easily detectable.

With this method it is possible to differentiate in a simple manner vascular smooth muscle cells from fibroblasts in a heterologous tissue culture.

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

Zusammenfassung. Die Differenzierung glatter Gefäßmuskelzellen von Fibroblasten in der Gewebekultur nach rein morphologischen Gesichtspunkten ist nicht möglich. Erst mit Hilfe der direkten Immunofluorescenz kann man schon am 1. Tag nach Kulturansatz glatte Gefäßmuskelzellen bzw. deren Vorläufer nachweisen. Mikrofilamente in Gefäßmuskelzellen treten ab 6.—10. Tag auf. Nach einer Inkubationszeit von 30 min bei Zimmertemperatur mit Antikörpern gegen Aktomyosin von glatten Muskelzellen sind Mikrofilamente in glatten Muskelzellen darstellbar. Fibroblasten hingegen zeigen bei gleichen Inkubationszeiten nur eine starke Kernfluorescenz, im Cytoplasma eine Primärfluorescenz aber *keine* Mikrofibrillen. Inkubiert man jedoch Fibroblasten *eine Stunde* bei 37° C mit Antiaktomyosin, dann lassen sich Mikrofibrillen leicht nachweisen.

Mit Hilfe dieser Methode ist es auf einfache Weise möglich, in einer heterologen Gewebekultur glatte Gefäßmuskelzellen von Fibroblasten sicher abzugrenzen.

The morphologic differentiation of vascular smooth muscle cells from fibroblasts in tissue culture is difficult if not impossible. We were able to observe (Hofmann and Goger, 1974) vascular smooth muscle cells in tissue culture with an applegreen fluorescence after one day in myocytoblasts which differentiated into myocytes between the 6th and 10th day. On the 10th day microfilaments could be demonstrated by the use of fluorescein-labelled antiactomyosin in the cytoplasm. After 30 minutes of incubation, in fibroblasts microfilaments could not be detected in the cytoplasm using antibody against smooth muscle cell actomyo-

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sin; we found only a faint primary fluorescence. In recent investigations Weber and Groeschel-Stewart (1974), as well as Lazarides and Weber (1974) were successful in visualizing both actin and myosin filaments (the so-called "attachment filaments" in nonmuscle cells). By immunofluorescence, microfilaments can be demonstrated in tissue culture in smooth muscle cells as well as in fibroblasts from the 6th to the 10th day, but the different types of cells cannot be distinguished. We therefore tried to work out a different behaviour respectively to the time of incubation.

Materials and Methods

Under sterile conditions, aortas were taken from 6- to 10-week old chickens. Intima, media, and adventitia were separated by stripping. For tissue culture only the media was cultivated in TC 199 + serum. The isolated cells were grown on coverslips at + 37° C in FALCON-Petri plates in a CO_2 incubator. The coverslips with vascular smooth muscle cells were fixed in ice-cold methanol on the 3rd, 4th, 5th, 6th, 8th, 10th, 11th, 14th, 17th, and 20th day. After treatment for 30 minutes in FITC-labelled antiactomyosin[1] in a humidified chamber at room temperature, coverslips were washed in PBS-buffer for 30 minutes. The cells were covered with glycine-buffer (pH 6.8) for fluorescence microscopy.

Additionally, coverslips with growth of vascular smooth muscle cells were fixed in glutaraldehyde on the 8th day and embedded and prepared in the usual manner for the electron microscopic examination.

Parallel to these experiments, three different cell strains of fibroblasts (33d passage of mouse calvarium, of L 929, passage 599/Sanford et al. and fibroblasts of mouse skin) were trypsinized and grown under identical conditions. Fibroblasts of calvarium and L 929 were fixed in ice-cold methanol on the 3d, 4th, 5th, 6th, 8th, 10th, 11th, 12th, 13th, and 18th day and incubated in antiactomyosin in a humidified chamber at room temperature for 30 minutes, washed in PBS for 30 minutes, and covered with glycine-buffer.

The fibroblasts of the mouse skin were cultivated under the same conditions and, according to the report by Weber et al. (1974) were fixed with 3.5% formalin/PBS for 20 minutes, washed in PBS, and again fixed in ice-cold methanol for 5 minutes. The cultures were incubated in antiactomyosin in a humidified chamber at + 37° C for one hour, washed in PBS for one hour and covered with glycine-buffer.

The preparations were examined under a LEITZ Ortholux fluorescence microscope using the filter combination BG 480 and K 510, an HBO 50 W lamp, and photographed with ILFORD HP 4 film.

Results

Distinct longitudinal microfilaments were discernible in the cytoplasm as early as the 6th day after the beginning of growth of vascular smooth muscle cells under the fluorescence microscope. The microfilaments also covered the nuclei (Figs. 1 and 2). The structure of the microfilaments can be observed at high resolution in the electron microscope on the 8th day. They are parallel to the cell membrane in a longitudinal direction (Fig. 3).

On the 8th day, the fibroblasts (calvarium and L 929) grow with a polymorphic structure (Fig. 4). There is a strong nuclear fluorescence after an incubation period of 30 minutes at room temperature, but there are no microfilaments (Fig. 5). Longitudinal microfilaments can be demonstrated in the fibroblasts of mouse skin on the 6th day after an incubation period of one hour at 37° C in antiactomyosin specific for smooth muscle cells (Fig. 6).

1 We are indebted to Prof. Dr. U. Stewart-Groeschel, Universitäts-Frauenklinik, Würzburg for preparation of FITC-labelled antiactomyosin.

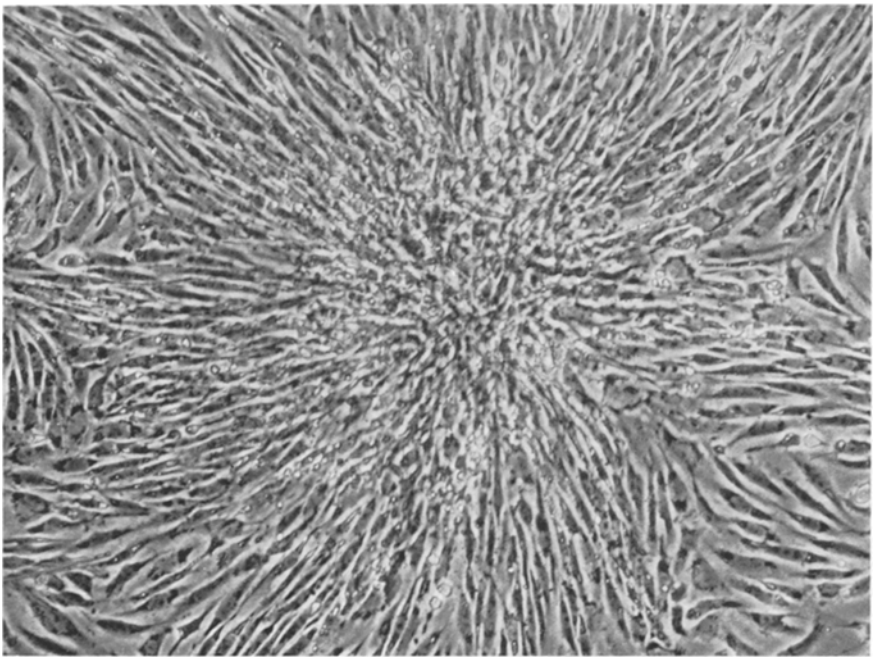

Fig. 1. Vascular smooth muscle cells of chicken after 8 days of tissue culture. Phase contrast, $\times 100$

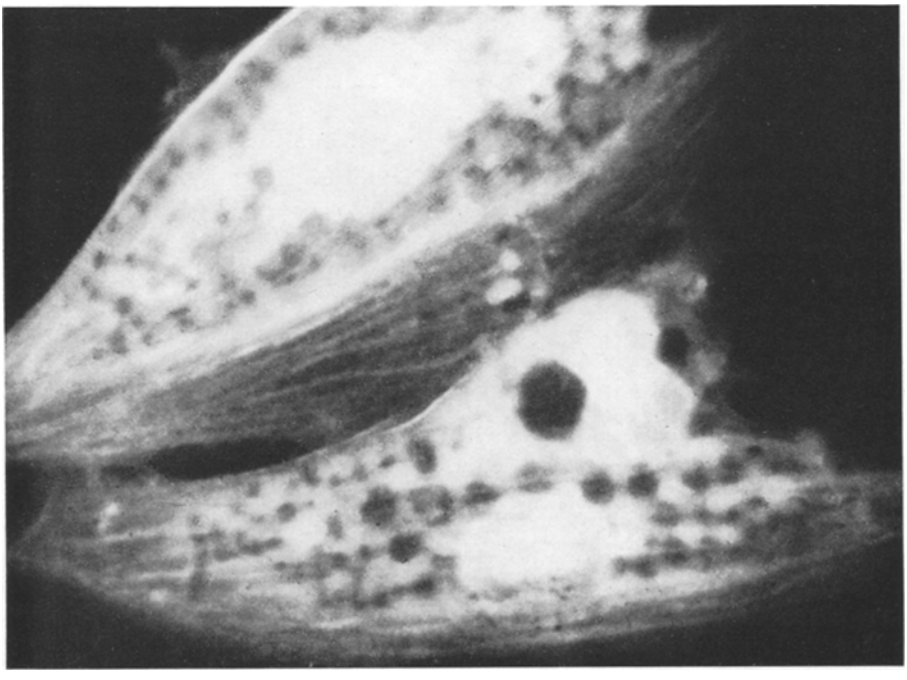

Fig. 2. Vascular smooth muscle cells of chicken after 11 days of cultivation. Longitudinal microfilaments are distinctly discernible. Fluorescence photograph after 30 minutes of incubation, approx. $\times 650$

Fig. 3. Vascular smooth muscle cell of chicken after 8 days of cultivation. Predominantly longitudinal microfilaments in the cytoplasm; nuclear membrane at the upper right hand corner. Electron-micrograph, approx. × 33,000[2]

Discussion

The morphologic differentiation of vascular smooth muscle cells and fibroblasts in tissue culture is difficult if not impossible. With the aid of direct immunofluorescence we were able to identify smooth muscle cells and their precursors as early as the first day.

Microfilaments were discernible in tissue culture during the 6th to the 10th day; in fibroblasts only a primary fluorescence could be detected, but no microfilaments (Hofmann and Goger, 1974. Becker and Nachmann, 1973) (Fig. 5). The results of Weber and Groeschel-Stewart (1974) as well as Lazarides and Weber (1974) indicate that the so-called "attachment filaments" can be visualized in the fibroblasts after treatment at 37° C in antiactin or antimyosin for a period of one hour.

Our earlier results (Hofmann and Goger, 1974) contradict those of Weber and Groeschel-Stewart as well as Lazarides and Weber (1974); this caused us to further investigate the visualization of microfilaments in fibroblasts and vascular smooth muscle cells by immunofluorescence. In numerous tests our results attained in 1974 were again confirmed, i.e. we found microfilaments only in vascular smooth muscle cells with an incubation period of 30 minutes in anti-

2 Our sincere appreciation to Prof. Dr. Franke, DKFZ (German Cancer Research Center), Heidelberg, for the electron-microscopical examination.

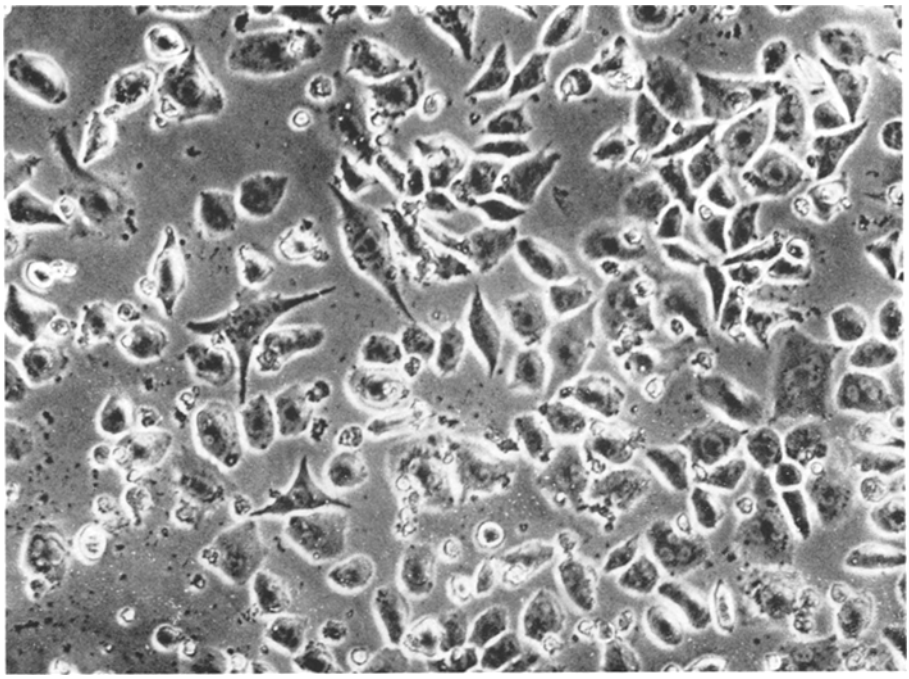

Fig. 4. Fibroblast tissue culture (L 929 mouse cell strain) after 8 days of cultivation. Phase contrast, × 100

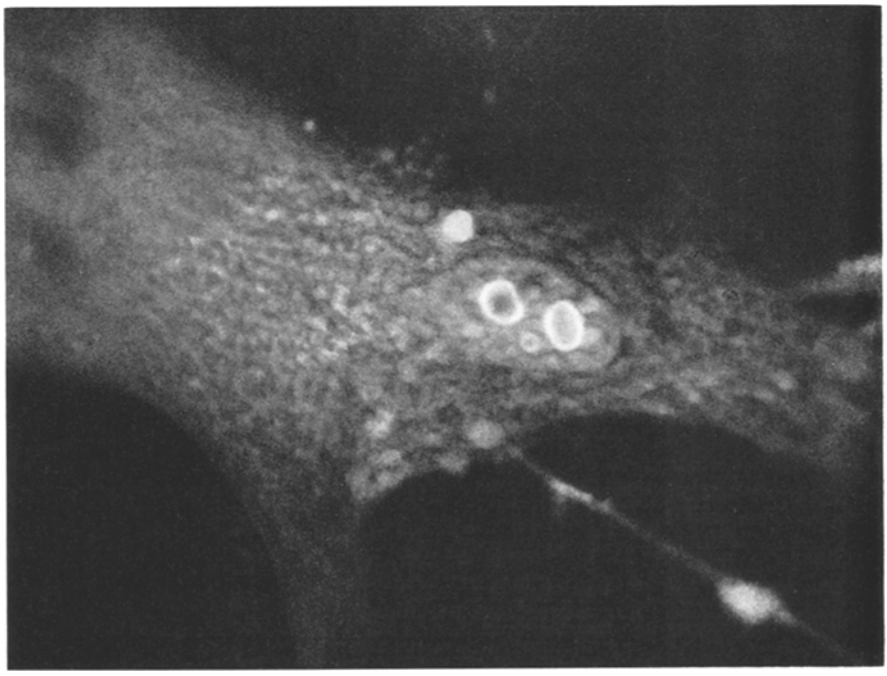

Fig. 5. Fibroblast (mouse calvarium) after 8 days of cultivation. No microfilaments, only faint secondary fluorescence in the cytoplasm; however, a strong nuclear fluorescence. Fluorescence photograph after 30 minutes of incubation, approx. × 650

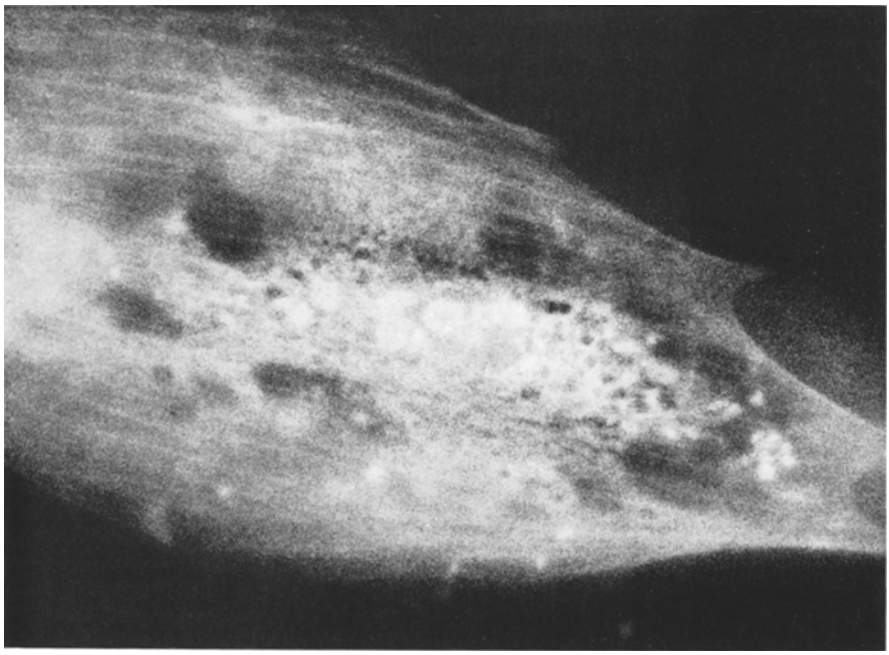

Fig. 6. Fibroblast (mouse skin) after 6 days of cultivation. Longitudinal microfilaments, strong nuclear fluorescence. Fluorescence photograph after one hour of incubation, approx. ×650

actomyosin at room temperature (Fig. 2), whereas only a faint primary fluorescence could be observed in fibroblasts (Fig. 5).

The results obtained by Weber and Groeschel-Stewart (1974) as well as by Lazarides and Weber (1974), however, could be fully confirmed (Fig. 6). Longitudinal microfilament structures, i.e. the so-called "attachment filaments", were found in fibroblasts after incubation for one hour at 37° C in antibody against smooth muscle cell actomyosin. These microfilaments—interpreted as contractile elements also in nonmuscle cells— are discernible when the incubation period of fibroblasts is doubled. Similar structures were found by Becker and Nachmann (1973) in endothelial cells and thrombocytes as well as by Groeschel-Stewart and Groeschel (1974) in mouse macrophages. We have the impression that the microfilaments are localized not only in the wall and adherent to the lower side of the cell, but also in the cytoplasm of the entire cell.

In comparing our results with those obtained by Weber and Groeschel-Stewart as well as by Lazarides and Weber (1974), we found that the apparent contradiction can be resolved by the simple change of the incubation period from half an hour to one hour, i.e. vascular smooth muscle cells can be differentiated from fibroblasts on the basis of microfilaments if incubated for a period of 30 minutes at room temperature. At this stage, no microfilaments are discernible in fibroblasts. However, if the incubation period is extended to one hour, microfilaments can be visualized in fibroblasts. Evidently this effect is caused by the fact that

the filaments of the vascular smooth muscle cells have a different structure compared to the fibroblasts and thus exhibit a different staining behaviour.

With this method it is possible to differentiate in a simple manner vascular smooth muscle cells from fibroblasts in a heterologous tissue culture.

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