KOREAN JOURNAL OF ZOOLOGY
Vol. 24, No. 4, 189-200 (1981)

Effects of Ultraviolet Irradiation on the Differentiation of Cultured Chicken Pectoralis Muscle Cells.

Hae-Moon Chung and Sang-Uk Nham
(Department of Biology, Seoul National University)

培養 鷄胚 筋細胞의 分化에 미치는 紫外線의 영향

鄭 海 文·南 相 旭
(서울大 師範大 生物學科)

(Received August 25, 1981)

요 약

12일간 배양한 계배에서 떼어낸 근모세포에 자외선을 조사하면 근육 분화에 심한 변화를 유발시킬 수 있다. 본 연구에서는 자외선이 세포분열 및 근섬유로의 전환, 근모세포와 근섬유의 형태에 미치는 영향을 조사하였다.

자외선을 받은 세포들은 크기가 작아지고 또 적은 수의 세포들이 근섬유 형성에 참여하기 때문에 근섬유의 직경이 좁아지고 길이 또한 작아진다. 자외선이 세포분열과 세포의 융합에 미치는 영향은 배양을 시작한 후 이른 시기에 조사할수록 그 효과가 크다. 또한 자외선의 양을 증가시키면 그 효과가 커져 지나친 양을 조사하면 세포에 치사작용을 나타낸다. 따라서 자외선에 의한 세포 밀도의 감소가 근섬유 형성의 저하를 초래하는 것으로 사료되어 이에 본 연구와 타 실험실에서 얻은 정보를 바탕으로 세포 융합 능력 감소의 원인에 대하여 토의 하였다.

INTRODUCTION

Differentiation of the embryonic skeletal muscle culture has long been recognized as a model system in which phenomena related to cell-cell interactions, membrane fusion and mechanisms of coordinate synthesis of proteins in eukaryotic systems can be studied (Holtzer, 1961; Konigsberg, 1963). Mononucleated myoblasts obtained from embryonic skeletal muscles, when cultured in a suitable medium, proliferate *in vitro* and subsequently to produce multinucleated myotubes. During the process of myoblast to myotube transformation a succession of more or less distinct phases of morphological and biochemical

This research was supported by a grant from the Korean Ministry of Education.

differentiation are described. In biochemical differentiation, the production of enzymes involved in muscle metabolism, and of contractile proteins are increased (reviewd by Buckingham, 1977).

The linkage between morphological and biochemical differentiation has been studied using two strategies. In the first strategy, fusion of myoblasts inhibited either by manipulating the culture medium or by using one of several inhibitors. There have been numerous reports that, particularly under conditions known to be unfavorable for cell fusion, such as low cell density or low concentration of Ca^{++} ions (Okazaki and Holtzer, 1965, 1966; Coleman and Coleman, 1968; Fambrough and Rash, 1971; Turner *et al.*, 1974). Another useful agents known to inhibit myotube formation are calcium specific chelator EGTA (Paterson and Strohman, 1972), thymidine analog 5-bromodexyuridine (Stockdale *et al.*, 1964), Ara-C (Doering and Fischman, 1974), and inhibitors of mitosis such as colchicine, actinomycin, fluorouracil and mytomycin (Okazaki and Holtzer, 1965).

In the second strategy, genetic studies of mutants or somatic cell hybrids which have lost the ability to fuse were carried out. Fusion-negative mutants of the L6 or L8 line of rat myoblasts can be readily obtained and exploited to examine the process of biochemical differentiation (Sanwal, 1979). The conclusion from both the biochemical and genetic studies is that fusion and muscle-specific protein synthesis are seperate phenomena and furthermore, they are regulated independently.

Ultraviolet irradiation has been used as a powerful tool to study the mechanism of cell differentiation and organogenesis in developing embryos (Chung and Malacinski, 1975; Malacinski *et al.*, 1977). UV damage to DNA is thought to result primarily in the formation of pyrimidine dimers, particularly those of thymine, within the strand of double helix. Other lights such as fluorescent light, X-irradiation are toxic and mutagenic to bacteria and mammalian cells *in vitro*. Since these radiations also induce breakage in DNA strands, cross-links and chromorsomal aberrations, they have been used in the study of the malignant transformation in cultured cells along other chemical carcinogens (Chan and Little, 1976; Kennedy *et al.*, 1980; Terzaghi and Little, 1976; Reznikoff *et al.*, 1973; Kakunaga, 1975).

However, the study on the effects of UV irradiation to myogenesis is very sparse. Hence, the present report describes the primary effects of UV irradiation on the cultures of chick embryonic pectoralis muscle cells as a preliminary investigation of the morphological and biochemical differentiation.

MATERIALS AND METHODS

1. Cell Culture

The primary cultures of myogenic cells from the breast muscle of 12 day old chick embryos were used. All cultures were plated at a normal density of 1×10^6 cells/ml

(2.5 ml on a coverslip in 50 mm dish), and maintained exactly same as described previously (Ha, 1979).

2. Ultraviolet Irrdiation

During the course of these experiments, an UVS-11 Mineral Light (San Gabriel, California) was employed and the output of the lamp was monitored continuously with an YSI-Kettering No 65 Radiometer. The output was calculated as much as 45 ergs/mm^2/sec.

At various time after plating, dishes were exposed at room temperature to UV (50 to 600 ergs/mm^2) after removing media and washing 2 times with Puck's phosphated saline solution. After irradiation, dishes were filled with fresh 8102 medium, and then incubated to 72 hours.

3. Cell Count

Cell count were made on cultures fixed with AFA and stained with Erlich's hematoxylin solution at a magnification of 400 with an Erma Microscope. Fusion percent was expressed as a ratio between the number of nuclei within multinucleated myotubes and within mononucleated cells. Overstaining or excessive overcrowding were rejected from the scoring. For each point, at least 10 randomly selected fields containing 1,000~2,000 cells of myotubes were counted.

RESULTS

1. Synchrony

Photomicrographs showing the progressive changes after plating the cells at 2.5×10^6 cells per dish are presented in Plate 1, and Figure 1 indicates the kinetics of cell proliferation and fusion of the myogenic cells based on the data in Table 1. It can be seen that the cell number was increasing very slowly during the first 32 hours *in vitro*. Note also the sparsity of myotubes or forming myotubes in the 40 hour control cultures, particularly when compared with identical plates at 48 hours of culture. However, there appeared a sharp increase during the next 16 hours (32-48 hours). After this period, there is no more increase in the cell number and keeps a plateau until 72 hour culture.

Table 1. Progress of myogenic cell fusion and number of cells per cover slip.

Culture Period(hrs)	24	28	32	36	40	44	48	52	56	60	64	68	72
Fusion %	5.0	—	9.9	11.3	12.6	24.8	39.1	61.2	—	62.6	—	—	54.4
Cell number($\times10^5$)	3.2	—	4.4	6.8	8.8	10.0	12.0	11.2	—	11.6	—	—	12.4

Meanwhile, myogenic cells come together in which the cells are aligned with their long axes parallel. There is a period of rapid fusion starting at 40 hours after seeding and this fusion burst is kept for the next 12 hours. Before this abrupt fusion increase, only 13% of the nuclei were found in multinucleated syncitia. By the time of maximum

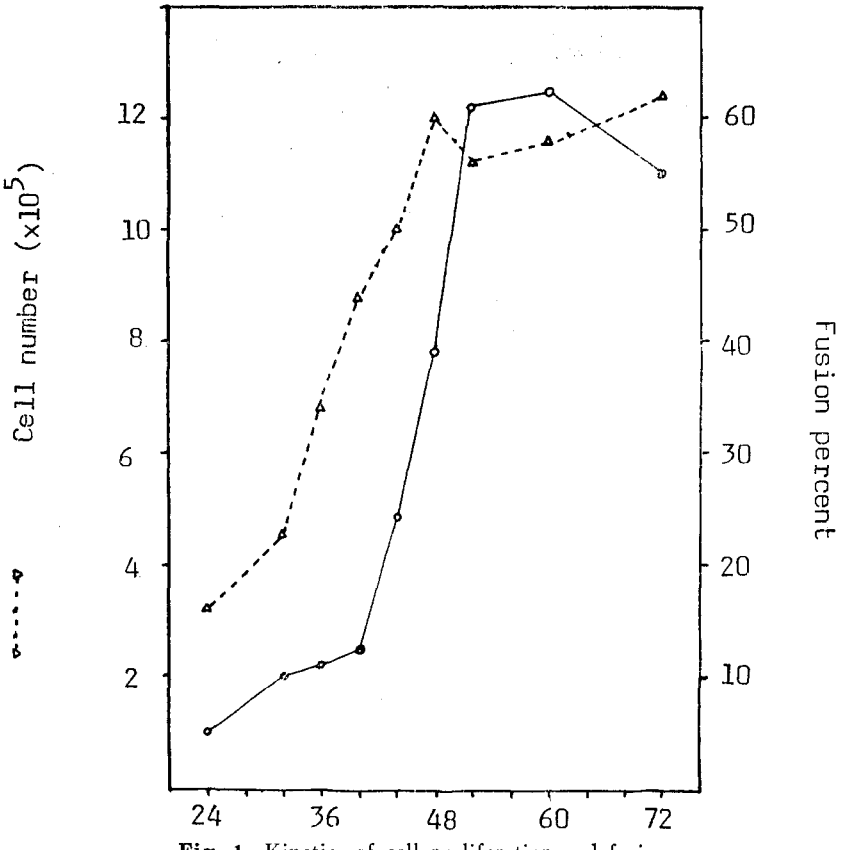


Fig. 1. Kinetics of cell proliferation and fusion

incorporation, however, about 60% of the myogenic cells have participated in myoutube formation. Thereafter, the proportion of nuclei in myotubes may increase at a low rate, or decrease, or remain constant up to 72 hours after seeding.

Comparing the periods of sharp increase in cell number and fusion, the data clearly indicates that cell divisions precede to the actual fusion under normal culture condition. Cell proliferation takes place several hours before the accumulation of the nuclei in myotubes.

2. Effects of UV Irradiation on the Cell Proliferation and Myogenesis

For the investigation of the UV effects, a study on the relationship between UV dose and cell number as well as fusion percent were carried out first. Thus, different amounts of UV ranging from 100 to 600 ergs/mm^2 were given at various time (30, 36, and 42 hours after seeding), and the cell number and the fusion percent were scored at 72 hour cultures.

As indicated in Table 2, there is almost no effect on the cell proliferation at a dose of 100 ergs/mm^2. Exposure to this amount of UV, cell number was increased as normal as

Table 2. Effect of UV dose on cell number 72 hr after seeding ($\times 10^5$ cells/cover slip)

UV'd time	Cell number at irradiation	UV dose									Control
		100	150	200	250	300	350	400	500	600	at 72hr.
30 hr	6.4	11.2	7.6	8.8	5.2	6.8	6.4	5.6	3.6	5.2	12.8
36 hr	6.8	10.4	12.0	7.6	7.6	7.6	7.2	5.2	4.4	4.0	12.8
42 hr	10.8	12.8	7.2	7.6	—	7.6	7.2	8.0	8.4	6.8	11.9

Cell number was determined by scoring cells at least 10 random spots in one cover slip. The values are means of 2 determinations.

Table 3. Effect of UV does on myogenesis. Fusion % at 72hr after seeding.

UV'd time	Fusion % at irradiation (control)	UV dose									Fusion % at 72hr (control)
		100	150	200	250	300	350	400	500	600	
30 hr	9.4	41.0	37.5	28.7	29.6	30.1	25.3	22.1	18.8	16.8	54.5
36 hr	13.4	41.5	38.1	36.6	32.1	31.7	25.8	22.3	16.6	13.9	54.5
42 hr	25.3	59.6	57.5	46.2	—	41.6	36.9	27.0	24.7	27.0	57.2

control cultures no matter when they were irradiated. However, responses to a dose of 150 ergs/mm^2 are different. Dishes irradiated at 30 and 36 hours, there was a slight increase in cell number from the time of irradiation. Dishes given with the same amount of UV at 42 hour, cell density was almost same as the above ones. But the absolute cell number was actually decreaseed from the value of 10.8×10^5 to 7.2×10^5 cells/cover slip. With increasing UV dose above 200 ergs/mm^2, there is a tendency of cell death.

Table 3. indicates the UV effect on cell fusion, and the same data are diagrammed in

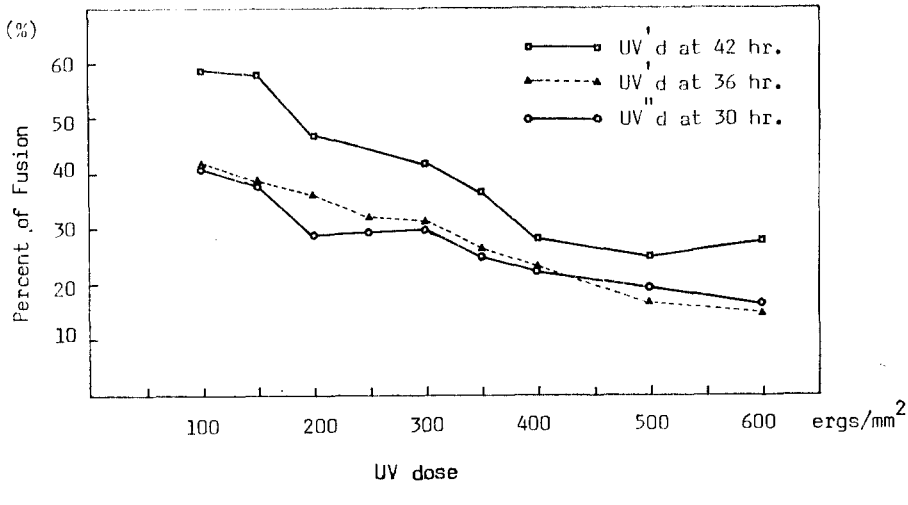


Fig. 2. Effect of UV dosage on myogenesis.

Figure 2 as kinetics of cell proliferation and fusion. The changing patterns of fusion percent are almost same when irradiated at 32 or 36 hours after plating. It should be noted that the effect on the myogenesis has already started to appear at a dose of 100 ergs/mm^2 The rate of myogenesis was about 75% of the control value. When dishes were irradiated at 32 or 36 hours with a does above 500 ergs/mm^2, there was practically no increase in the fusion percent comparing with the values of the time of irradiation. But the dose effect is somewhat different if UV were given at 42 hour cultures. No effect can be detected up to 150 ergs/mm^2. From 200 ergs/mm^2, the inhibiting action to the myogenesis was in reverse proportion to the UV dose. In addtion, no further fusion occured after irradiation with a does above 400 ergs/mm^2.

Table 4. Effect of UV on myogenesis (Time course)

Irradiation time (hrs after seeding)	30	32	36	40	42	44	48	52	56	60	64	68	72
Fusion (%)	32.2	32.6	36.0	38.4	50.7	45.4	46.2	53.7	52.8	—	—	—	53.5
Cell number($\times 10^5$)	7.6	7.6	6.8	8.4	7.5	8.0	8.0	12.0	11.2	14.0	—	—	16.0

Cell are exposed to 180 ergs/mm^2 dose of UV at each time and incubated till 72 hr after seeding when scored cell number and percent of myogenesis

Table 4 shows a time course of the effect on the cell division and fusion with a certain amount of UV. A dose of 180 ergs/mm^2 was adopted since the previous data in Table 2 and 3 revealed that cell proliferation and fusion were affected with 150 ergs/mm^2. Thus, dishes were exposed to UV from 30 to 60 hours after seeding, and the cell number and the fusion percent were scored at 72 hour cultures. With this amount of UV, dishes displayed about same number of cells if they were irradiated earlier than 48 hours after seeding. In other sense, this also means that the actual cell number at 72 hour in the irradiated dishes (8×10^5 cells/cover slip) has decreased comparing with those values of non-irradiated control at each irradiation time. It is also apparent that the inhibition to mygenesis is more effective if cells are irradiated at earlier stages. When the initial cell number and the fusion percent at 30 and 42 hour cultures are compared, it can be clearly seen that the myogenic events have been progressing without cell proliferation. If cells were irradiated later than 52 hours, there was no further effect on either the cell number or the fusion.

3. UV Effects on the Morphology

Plate II contains photographs of cells which were irradiated at 30 hours after seeding and the pictures are taken at 72 hour cultures. Total cell number and cells participating in the myotube formation became fewer and fewer with increasing UV dose. Morphology of the irradiated cells and of the myotubes are quite abnormal. Irradiated cells are much smaller in size, possibly due to loss of the cytoplasm or cell fragmentation. Size of myotubes is also smaller since only few cells are comprising them. Furthermore, myotubes

look like a string with a narrow width.

DISCUSSION

The pattern of growth and fusion kinetics observed in this report shows a dramatic enhancement within short period after inoculation. This is a typical manifestation of muscle cell differentiation *in vitro*, and well agreed with those results obtained by the other investigators (O'Neill and Stockdale, 1972; Paterson and Strohman, 1972; Doering and Fischman, 1974).

As revealed in Table 2, cell division is not affected by irradiation under a certain amount of UV (100 ergs/mm^2). On the other hand, excessive dose causes cell death which results in a drastic decline in the cell number at 72 hour cultures. In case of no change in cell density from the time of irradiation to 72 hour, it might be a result of a balance between cell death by the irradiation and cell proliferation, not by complete arrest of cell division after irradiation. UV effect on the cell number is displayed most effectively during the period of exponential growth (32-48 hours after seeding) and thereafter such effect can not be observed (Table 4). The fact that the earlier irradiation induces severer inhibition to cell fusion is clearly displayed in the line of 100 ergs/mm^2 in Table 3 because the fusion percents of 30, 36, and 42 hour irradiation are 41%, 41.5% and 59.6%, respectively. As well, given with a same does of 180 ergs/mm^2, the inhibiting action to fusion is more striking when irradiated at earlier stages. However, this effect disappears from 42 hour cultures which correspond to the beginning of the fusion burst.

Above observations strongly suggest that the UV irradiation does not affect directly to the process of fusion itself once myoblasts aquire the capacity for fusion. In other words, there seem to be no direct influence on the events which occur later than DNA replication such as cell-cell recognigion, alignment of myogenic cells and formation of syncitia. This is well agreed with Doering and Fischman's results where they observed a fusion delay if Ara-C was added to the medium 6 hours earlier than the normal fusion burst but not later than that time. However, it does not imply that UV irradiation has no influence to the process of fusion itself because cell fusion was inhibited with a low dose which has no effect on the cell proliferation (Table 2). The inhibition may not be a result of interference with DNA replication but by some direct or indirect influences which cause alterations in the cell membrane (see below). If this assumption is correct, dose effects to the DNA replication and to the other cytoplasmic components are different.

One of the remarkable effects of UV irradiation is lowering the fusion percent. This is posssibly due to inhibition of DNA replication by forming thymine dimer. Earlier works have revealed that the onset of fusion is delayed by lowering the initial cell density (Morris and Cole, 1972; Doering and Fischman, 1974). The induction of low cell density by UV irradiation should lead to decrease cell fusion.

Another possible reason for the lowering fusion percent is changes in Ca^{++} ions level and in the cell membrane. It is a well known fact that Ca^{++} ion is an absolute prerequisite for cell adhesion. In his study of the UV effects on the proliferation and migration of primordial germ cells of the amphibian embryo, Dixon (1981) has observed that the UV irradiation affects on Ca^{++} ions level in the cytoplasm and the alteration in Ca^{++} levels leads secondary and tertiary effects to the cell such as changes in the flux of other ions, particularly Na^{+} and K^{+}. The cumulative results should be a derangement of the normal process of biochemical events. Therefore, it is not dangerous to assume that the cell fusion is delayed by the same kind of disturbance in normal process in the cytoplasm which, in turn, alter the rate of cell division and cell adhesion. This assumption is strengthened by the observations that the cell density was reduced in the irradiated region of the fertilized amphibian eggs due to the release of Ca^{++} ions from cytoplasm storage areas and disturbance of other ionic conditions (Morrill *et al.*, 1971; Malacinski *et al.*, 1977). Thus, the overall changes in biochemical process are expressed as the abnormal morphology of individual cells and the shape of myotubes. With increasing dose of UV, a drastic alterations in the cytoplam may cause cell death which, in turn, decrease the number of cells participating in the myotube formation.

Another interesting result observed in the present study is the fact that the fusion of myotubes was progressing without enhancement of cell density. As disclosed in the time course or UV effect, fusion percent has increased even though the actual cell number has not changed (see up to 48 hours in Table 4). This is more easily understandable when the date in Table 1 and 4 are considered together. Considering the values of 36 hour cultures, for example, the cell number at 72 hour has not changed from the time of irradiation (6.8×10^{5}). However, the original fusion percent of 11.3% at irradiation has increased to 36% at 72 hour after plating. This increased fusion percent with same or lower cell density can be observed up to irradiation at 48 hour cultures. This phenomenon, suggesting that muscle cell fusion is independent of the enhancement of cell number, was also observed by Bischoff and Holtzer (1969) that some cells fuse without ever having divided *in vitro*. Turner *et al.* (1976) too, recognized that fusion itself can occur in the absence of cell division.

In view of the results presented in this report, and the findings from other laboratories discussed above, it will now be of significance to characterize the mode of UV action to DNA and to membrane by studying biochemical process and morphology in detail.

SUMMARY

Drastic alterations in myogenesis could be induced by ultraviolet irradiation of the myogenic cells derived from 12 day old chick embryo skeletal muscle. The effects of irradiation on various aspects, including cell division, transformation to myotubes, and morphology of

myoblasts and myotubes, were examined.

Irradiated cells were smaller in size, and only few cells transformed resulting in smaller size of myotubes with a narrow width. Both the inhibiting actions to cell division and to fusion were more striking when irradiated at earlier stages after plating. As well, cell division and fusion were inhibited more effectively with increasing UV dose and excessive amount caused cell death. A lowering cell density was thought to account for the decrease in myogenesis and possible reasons for the decrease in the capacity for fusion were discussed in view of the results presented in this report and of the findings from other laboratories.

REFERENCES

Bischoff, R. and H. Holtzer, 1969. Mitosis and the processes of differentiation of myogenic cells *in vitro*. *J. Cell Biol.* **41**:188-200.

Buckingham, M.E., 1977. Muscle protein synthesis and its control during the differentiation of skeletal muscle cells *in vitro*. In: biochemistry of cell differentiation II. (J. Paul editor). University Park Press, Baltimore, Vol. **15**:269-332.

Chan, G.L. and J.B. Little, 1976. Induction of oncogenic transformation *in vitro* by ultraviolet light. *Nature* **264**:442-444.

Chung, H-M. and G.M. Malacinski, 1975. Repair of ultraviolet irradiation damage to a cytoplasmic component required for neural induction in the amphibian egg. *Proc. Nat. Acad. Sci.* USA **72**: 1235-1239

Coleman, J.R. and A.W. Coleman, 1968. Muscle differentiation and macromolecular synthesis. *J. Cell Physiol.* **72**:Suppl. 1. 19-34.

Dixon, K.E., 1981. The origin of the primordial germ cells in the amphibia. In press

Doering, J.L. and D.A. Fischman, 1974. The *in vitro* cell fusion of embryonic chick muscle without DNA synthesis. *Develop. Biol.* **36**:225-235.

Fambrough, D. and J.E. Rash, 1971. Development of acetylcholine sensitivity during myogenesis. *Develop. Biol.* **26**:55-68.

Ha, D.B., R. Boland, and A. Martonosi, 1979. Synthesis of the calcium-transport ATPase of sarcoplasmic reticulum and other muscle proteins during development of muscle cells *in vivo* and *in vitro*. *Biochim. Biophys. Acta* **585**:165-187.

Holtzer, H., 1961. In molecular and cellular structure. 19th Growth Symposium. O. Rudnick, editor. The Ronald Press Company, New York. 53.

Kakunaga, T., 1975. The role of cell division in the malignant transformation of mouse cells treated with malignant transformation of mouse cells treated with 3-methylcholanthrene. *Cancer Res.* **35**:1637-1642.

Kennedy, A.R., M.A. Ritter, and J.B. Little, 1980. Fluorescent light induced malignant transformation in mouse embryo cell culture. *Science* **207**:1209-1211.

Konigsberg, I.R., 1963. Clonal analysis of myogenesis. *Science* **140**:1273.

Malacinski, G.M., A.J. Brothers and H-M. Chung, 1977. Destruction of components of the neural

induction system of the amphiblan egg with ultraviolet irradiation. *Develop. Biol.* **56**:24-39.

Morrill, G.A., A.B. Kostellow and J.B. Murphy, 1971. Sequential forms of ATPase activity correlated with changes in cation binding and membrane potential from meiosis to first cleavage in *R. pipiens. Exptl. Cell. Res.* **66**:289-298.

Morris, G.E. and R.J. Cole, 1972. Cell fusion and differentiation in cultured chick muscle cells. *Exptl. Cell Res.* **75**:191-199.

Okazaki, K. and H. Holtzer, 1965. An analysis of myogenesis *in vitro* using fluorescein labeled antimyosin. *J. Histochem. Cytcchem.* **13**:726-739.

Okazaki, K. and H. Holtzer, 1966. Myogenesis fusion, myosin synthesis and the mitotic cycle. *Proc. Nat. Acad. Sci.* USA **56**:1484-1490.

O'Neill, M.C. and R.C. Strohman, 1969. Change in DNA polymerase activity associated with cell fusion in culture of embryonic muscle. *J. Cell Physiol.* **73**:61-68.

O'Neill, M.C. and F.E. Stockdale, 1972. A kinetic analysis of myogenesis *in Vitro. J. Cell Biol.* **52**:52-65.

Paterson, B. and R.C. Strohman, 1972. Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. *Develop. Biol.* **29**:113-138.

Reznikoff, C.A., J.S. Bertam, D.W. Brankow, and C. Heidelberger, 1973. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* **33**:3239-3249.

Sanwal, B.D., 1979. Myoblast differentiation. TIBS. July 155-157.

Stockdale, F., K. Okazaki, M. Nameroff, and H. Holtzer, 1964. 5-Bromodeoxyuridine: Effect on myogenesis *in vitro. Science.* **146**:533-535.

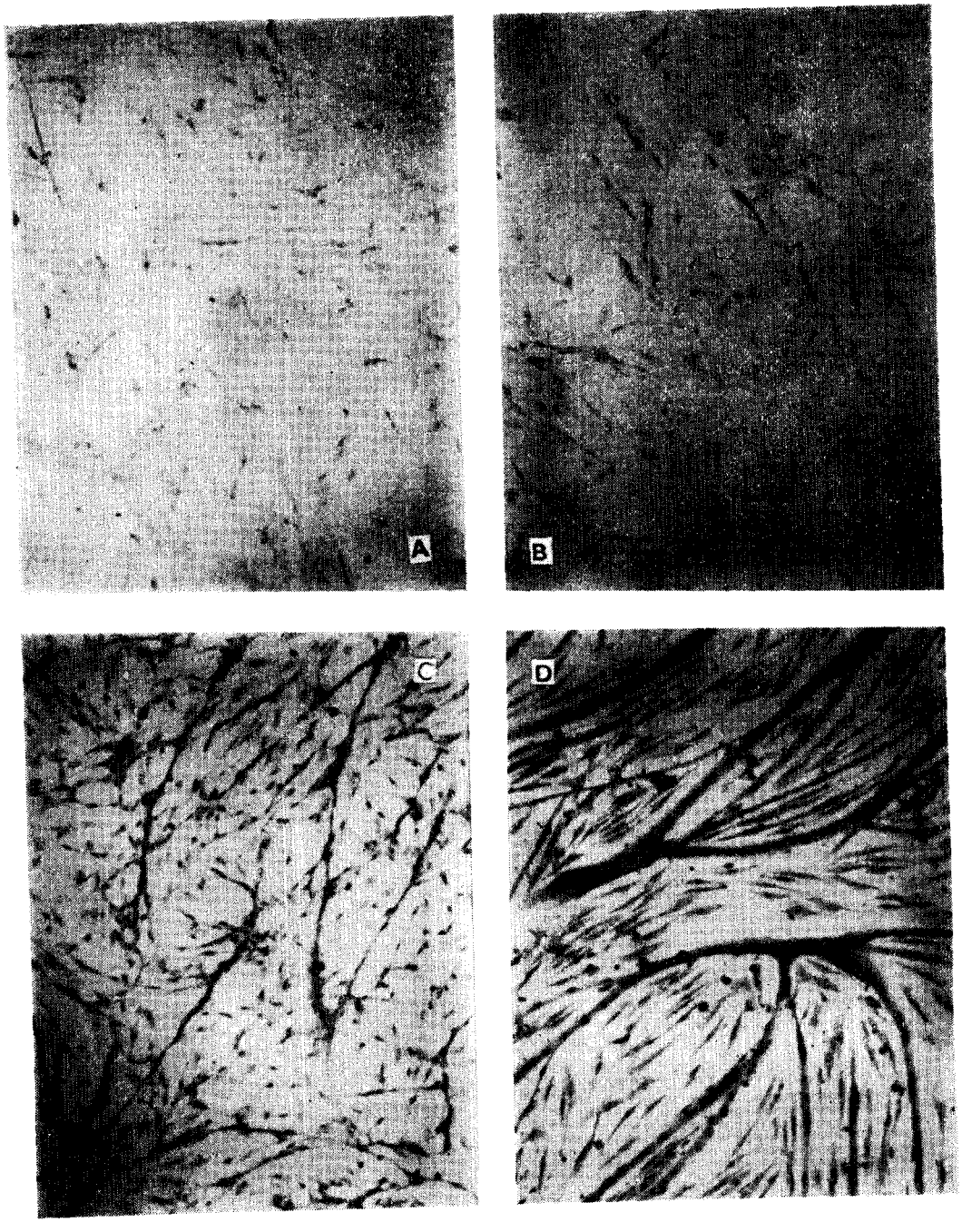
Stockdale, F.E., and M.C. O'Neill, 1972. Deoxyribonucleic acid synthesis, mitosis and skeletal muscle differentiation. *In vitro* **8**:212-225.

Terzaghi, M. and J.B. Little. 1976. X-radiation induced transformation in a C3H mouse embryo derived cell line. *Cancer Res.* **36**:1367-1374.

Turner, D.C., V. Maier, and H.M. Eppenberger, 1974. Creatin kinase and aldolase isozyme transitions in cultures of chick skeletal muscle cells. *Develop. Biol.* **37**:63-89.

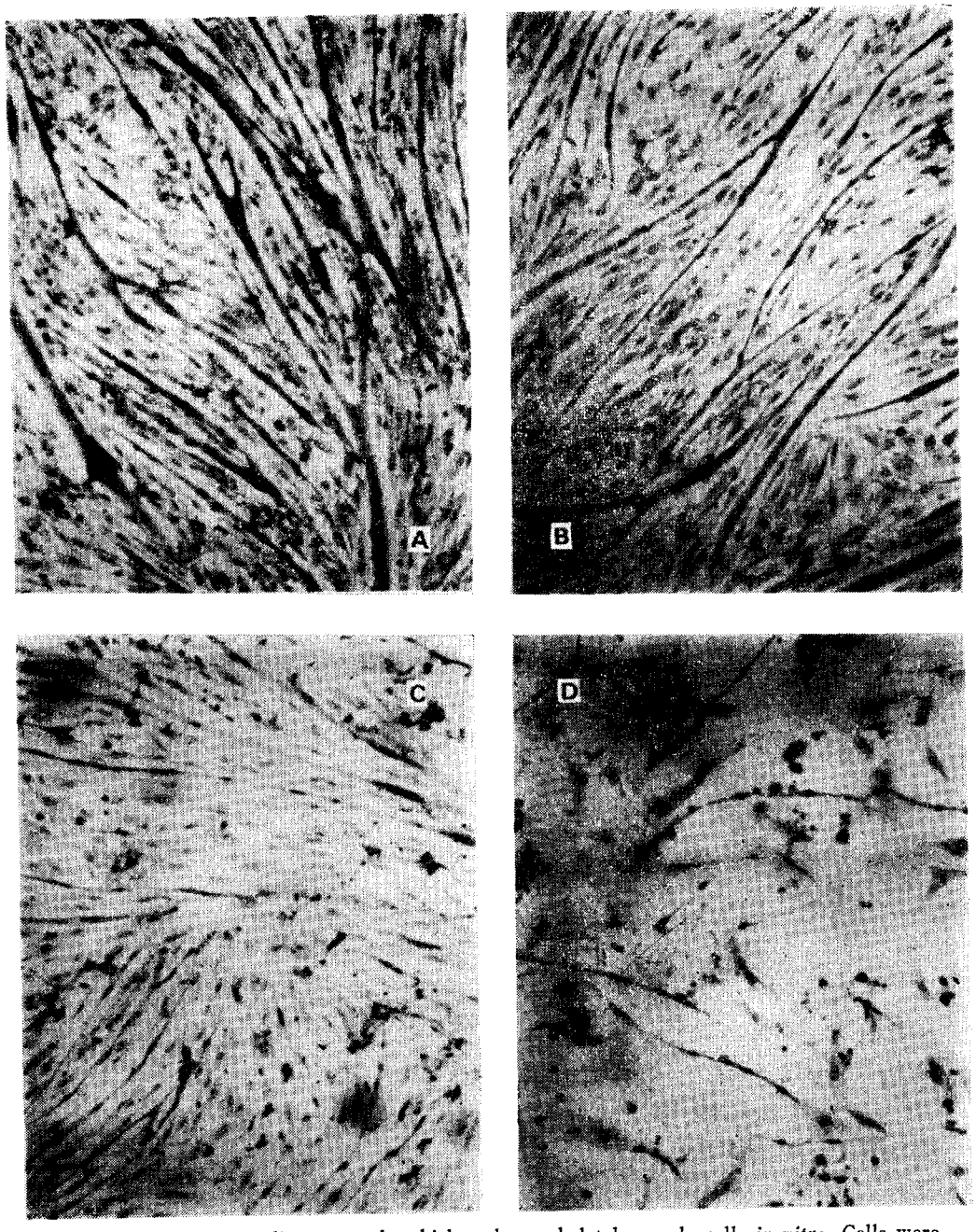
Turner, D.C., R. Gmür, M. Giegrist, E. Burckhardt, and H.M. Eppenberger, 1976. Differentiation in cultures derived from embryonic chick muscle. *Develop. Biol.* **48**:258-283.

Plate I



Progressive changes after plating the cells derived from 12 day chick embryo skeletal muscle
A: at 24 hr, B: at 36 hr, C: at 48 hr, D: at 72 hr. ×100.

Plate II



Dose effect of UV irradiation on the chick embryo skeletal muscle cells *in vitro*. Cells were irradiated at 30 hr cultures with various amount of UV and the pictures were taken at 72 hrs after plating.

A: 50 ergs/mm^2, B: 150 ergs/mm^2, C:300 ergs/mm^2, D: 600 ergs/mm^2. ×100.