Acta orthop. scand. 46, 161–176, 1975

Department of Orthopaedic Surgery, Kanazawa University Medical School,
Kanazawa City, Japan.

CYTOKINETIC ANALYSIS OF OSTEOGENIC CELLS IN THE HEALING PROCESS AFTER FRACTURE

SHOHEI MANABE, IWAO SHIMA & SHIRO YAMAUCHI

Accepted 21.x.74

Investigating the growth of long bones, Walker et al. (1972 a, b), Kemper (1971), Tonna (1958), Tonna & Cronkite (1961, 1962) have been studying chondrocytes and periosteal cells autoradiographically. Tonna & Cronkite (1961) studied post-fracture cellular response autoradiographically and proposed the required conditions for the occurrence of osteogenesis.

We have pursued the study of precursors of osteoblast and cartilage cells during the reintegration period after a fracture by labelling with H^3-Thymidine, S^{35}, and H^3-Proline and have made special studies on the process of differentiation of mesenchymal-like cells and analyzed the cytokinetics of these cell systems.

EXPERIMENTAL MATERIALS AND METHODS

The femurs of 14-day-old ddN male mice were fractured subcutaneously. One and 2 weeks after fracture, H^3-Thymidine, S^{35}, and H^3-Proline were administered and the fractured parts were dissected out at certain time intervals and were examined microautoradiographically using the dipping method.

Cumulative labelling of H^3-Thymidine

H^3-Thymidine 1.0 μCi/g was injected at 3-hour intervals into the abdominal cavity of the mice 1 and 2 weeks after fracture. Injection was repeated up to 16 times, until 45 h had elapsed. The fractured parts were dissected out 2 and 3 h after injection and every 3 h thereafter. Then, specimens taken within a 48-hour period were examined. After fixation of the specimens in Carnoy's solution they were electrically decalcified by 10 per cent trichloroacetic acid solution and were embedded in paraffin. Finally, 6 μ sections were prepared.

Uptake of H^3-Proline and S^{35}

H^3-Proline 2.0 μCi/g was injected into the abdominal cavities of a group of mice 1 and 2 weeks after fracture and likewise S^{35} 1.0 μCi/g was injected into another

group by the same method. The fractured parts were dissected out at intervals of 1, 5, 10, 20, 30 min and 2 h. Using the same method as with the H^3-Thymidine group, 6 μ paraffin sections were prepared.

Time-wise changes of H^3-Thymidine uptake

After producing fractures in the mice by the same method as above, H^3-Thymidine 1.0 μCi/g was injected into their abdominal cavities on the 3rd, 5th, 7th, 10th and 14th days after fracture. The fractured part was dissected out 2 h after such injection and fixed in Carnoy's solution. The time-wise changes after fracture were observed using the 2-hour Labelling Index.

Dipping method

DNA staining of these prepared sections was carried out by the Feulgen reaction. Thereafter the specimens were subjected to dipping, using "Sakura emulsion NR-M_2 for autoradiography". The specimens were then placed in a sealed box with a tube containing silica gel and exposed for 30 days at 4° C. After the specimens were developed they were studied microscopically.

Measurement of precursor-uptake in matrix

Uptake of H^3-Proline and S^{35} into cartilage and osteoid matrix was measured as the number of grains per unit area. Using a micrometer and gradated viewing lens, the zone line was set at each detection section and the number of grains contained in each zone was counted. The number of grains per unit area of matrix (1 mm^2) was calculated from this information.

Analysis of cumulative labelling

As for the test *in vivo*, relative cumulative results are obtained by injecting H^3-Thymidine at 3 hourly intervals. Labelled cells were counted with the specimens obtained by dipping and the percentage of labelled cells (P.L.C.) was calculated. When time is taken as the abscissa and the P.L.C. is plotted on the ordinate axis as indicated in Figure 8, a linear increase of P.L.C. is shown on the graph. When the distance between point "b" on the X axis, which represents the point where the above-mentioned straight line reaches 100 per cent, and point "c", at which the extension of the said straight line cuts through the X axis, is measured the generation time (Gt) is obtained. However, it is a well-known fact that the P.L.C. *"in vivo"* never reaches 100 per cent. The result shown in Figure 9 suggests that there exists (100-d) per cent of undivided cells. The value "a" of P.L.C. at time $t = 0$ indicates a Labelling Index (L.I.) but DNA synthetic time (St) satisfies the equation.

$$C : Gt = 100 \times \frac{St}{Gt} : 100$$

Therefore it is understood that St is represented by the measured value of "c". Postsynthetic resting time or premitotic time (Gt_2) is the time from injection of H^3-Thymidine to the time of the first appearance of labelled mitotic cells. Thus the mitotic time (Mt) can be calculated from the equation.

$$\text{Mitotic Index (M.I.)} = 100 \times \frac{Mt}{Gt}$$

Using these values, presynthetic resting time (Gt_1) is calculated by the following equation.

$$Gt_1 = Gt - (Gt_2 + Mt + St)$$

EXPERIMENTAL RESULTS

Uptake of H^3-Proline in the fractured parts

One week after fracture: Five min after the injection of H^3-Proline, the grains were observed in osteoblasts and proliferative cartilage cells and a time-wise change of the number of grains in each cell was noticed. After 2 h, the average number of grains in the cells decreased as shown in Figure 1. Similar findings were observed in hypertrophic cartilage cells. As indicated in Figure 1, the average intracellular number of grains reached its maximum, and thereafter grains were gradually discharged outside the cells to the matrix. Two hours after injection, it was observed that grains had migrated into the matrix. The number of grains was 2600/mm² in the matrix of the osteoid, 670/mm² in the proliferative cartilage and 480/mm² in the matrix of the hypertrophic cartilage. It was characteristic for the matrix of the osteoid that grains were densely lined in a belt form at the surface of the osteoid (Figure 2).

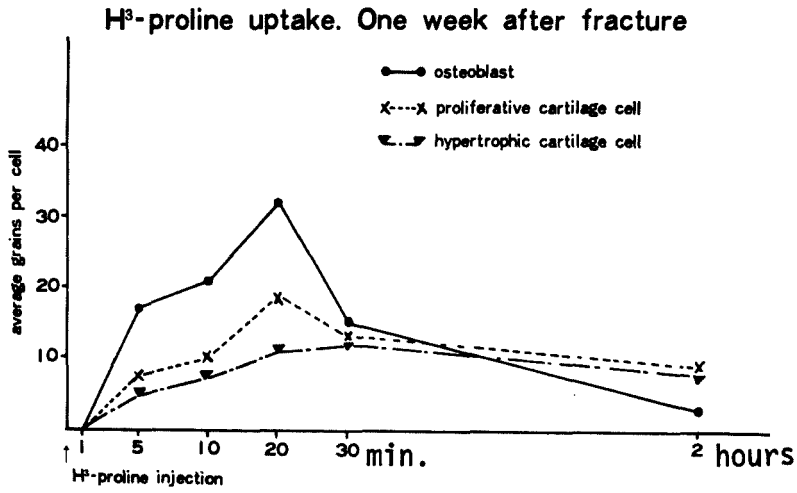

Figure 1. The intracellular number of grains of H^3-Proline per cell.

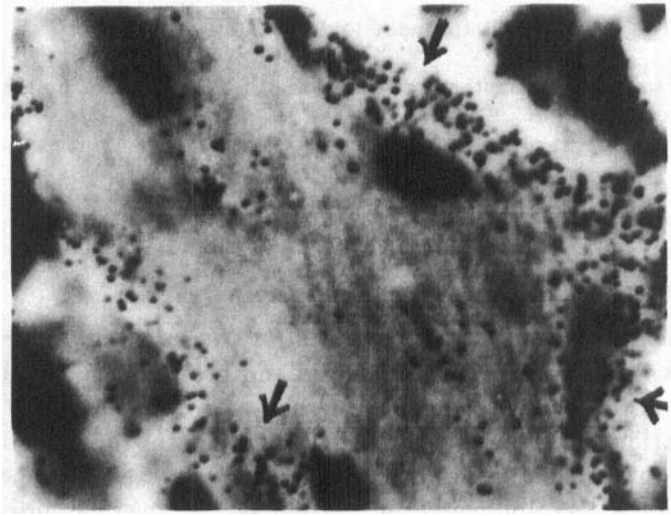

Figure 2. Many grains of H^3-Proline are densely lined in a belt form at the surface of the osteoid. One week after fracture and 2 h after the injection of H^3-Proline. × 1000.

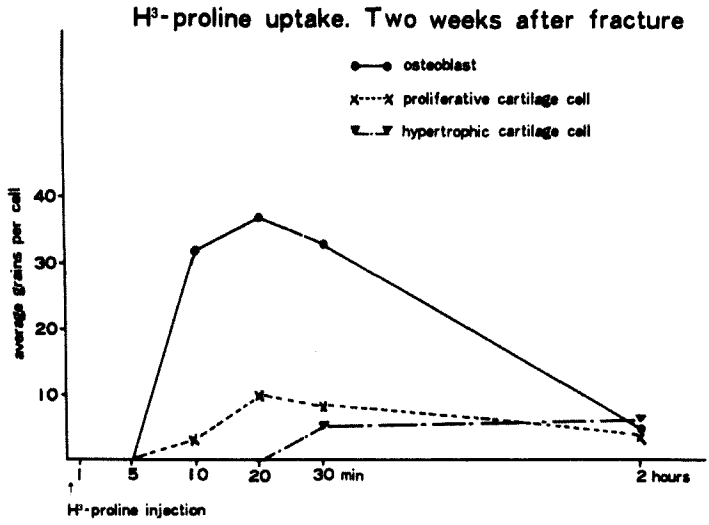

Figure 3. The average number of grains of H^3-Proline per cell.

Two weeks after fracture: Ten min after injection, grains appeared in osteoblasts and in the proliferative cartilage cells. As shown in Figure 3, the intracellular number of grains reached its maximum 20 min after injection for both systems. In the case of hypertrophic

cartilage cells, grains were first observed 30 min after injection. By this time, however, the grains in other cellular systems had already migrated into the matrix of the osteoid. Two hours later, the average number of grains in the matrix of the osteoid was 1100/mm², lined in a belt form, 85/mm² in the proliferative cartilage zone and 53/mm² in the hypertrophic cartilage zone.

Uptake of S^{35} in the fractured parts

One week after fracture: Five min after administration of S^{35}, labelling was observed in the matrix of cartilage and proliferative cartilage cells and, 10 min later, intracellularly in the hypertrophic cartilage cells. Thirty min later, the intracellular number of grains reached a maximum but even 2 h later, the grains were still observed in both cartilage cell systems (Figure 4). However, as early as 20 min after injection of S^{35}, grains from the cells migrated to the matrix of the cartilage. After a further 30 min, the migration became quite vigorous and 2 h later, the average number of grains was 1960/mm² in the matrix of the proliferative zone and 620/mm² at the hypertrophic zone. In spite of the fact that no grains were observed in osteoblasts during this 2-hour period, an average number of grains of 260/mm² was found in the osteoid matrix 2 h later.

Two weeks after fracture: The grains first appeared in the matrix

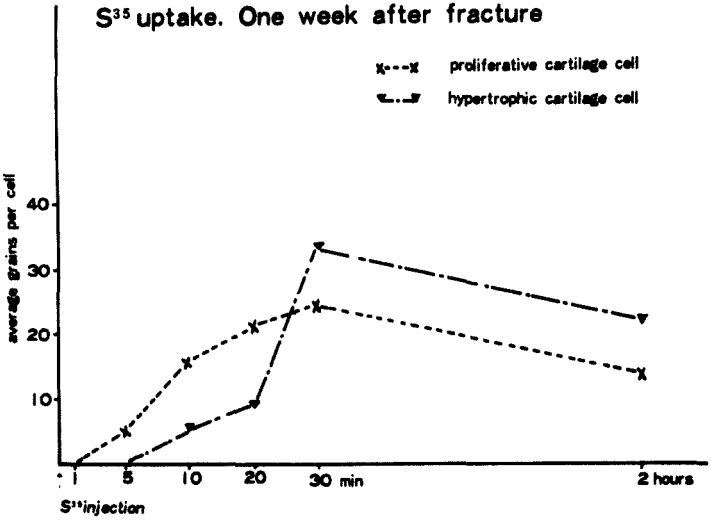

Figure 4. The average number of grains of S^{35} per cell.

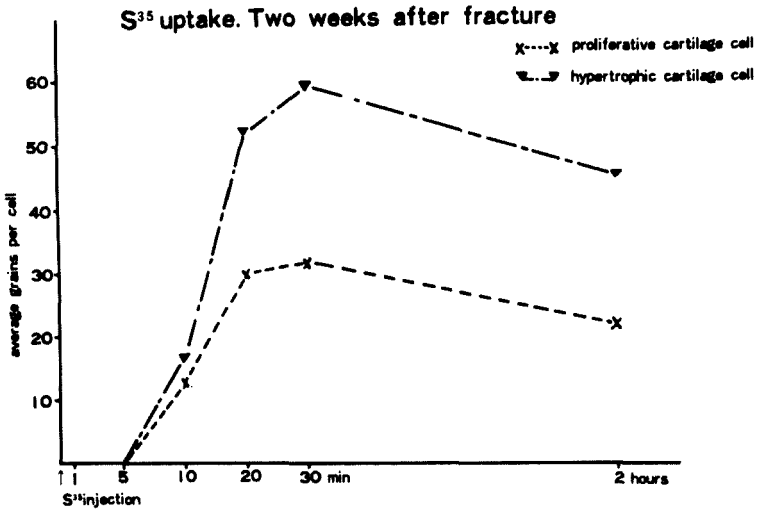

Figure 5. The average number of grains of S^{35} per cell.

of the cartilage 5 min after injection of S^{35} and 10 min later intracellular grains were observed. As indicated in Figure 5, 30 min after injection the intracellular number of grains reached a maximum in both the proliferative cartilage cells and the hypertrophic cartilage cells. Even after 2 h a considerable number of grains was still observed. Migration of grains into the cartilage matrix started 20 min after injection of S^{35}, and after 2 h the average number of grains in the proliferative zone had increased to 1970/mm^2 while that in the hypertrophic zone was 1120/mm^2. Also in the matrix of the osteoid, 820 grains/mm^2 were observed but there was no uptake of S^{35} in osteoblasts during the observation period.

Uptake of H^3-Proline and S^{35} in mesenchymal-like cells

Mesenchymal-like cells which appeared after fracture gave an image suggesting morphological transformation of mesenchymal-like cells into the osteoid tissue or the cartilage tissue. At both zones, the uptake of H^3-Proline was observed, but intracellular uptake of H^3-Proline and S^{35} in the two transitional zones was different quantitatively. Thus, as regards mesenchymal-like cells in the region where transformation into osteoid tissue was taking place, a marked uptake of H^3-Proline was observed whereas the same transitional zone to cartilage tissue indicated an even more marked intracellular uptake of S^{35} (Figure 7).

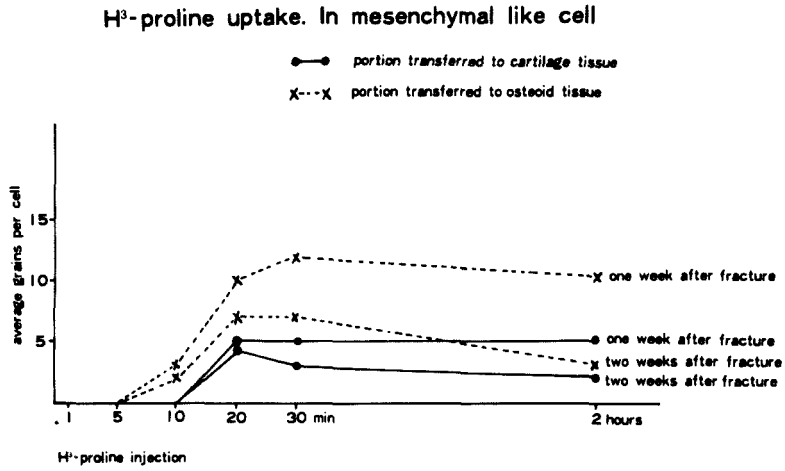

Figure 6. The average number of grains of H³-Proline per cell.

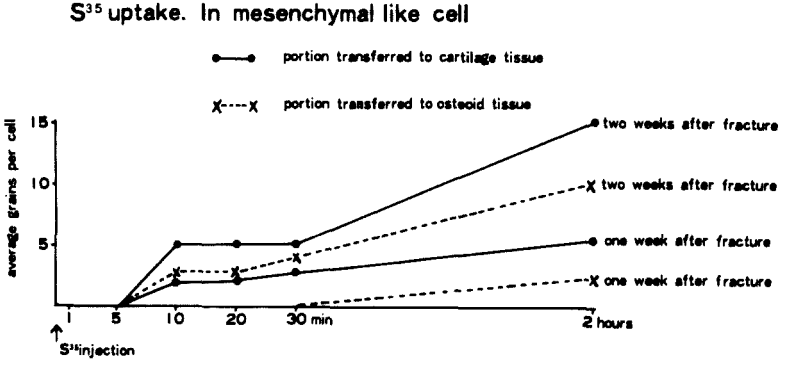

Figure 7. The average number of grains of S³⁵ per cell.

Analysis of cytokinetics

Analysis of the cytokinetics was made by cumulative labelling using H³-Thymidine on three cellular systems, viz. mesenchymal-like cells, cartilage cells and osteoblasts.

Analysis one week after fracture. In the case of mesenchymal-like cells, 2 hours after starting the administration of H³-Thymidine, the P.L.C. was 15 per cent, as illustrated in Figure 10, and thereafter as time went by the P.L.C. increased almost in a straight line, reaching 90 per cent in 21 h, and thereafter the curve formed a plateau. From Figure 10, it is known that one week after fracture, Gt was 23 h and St

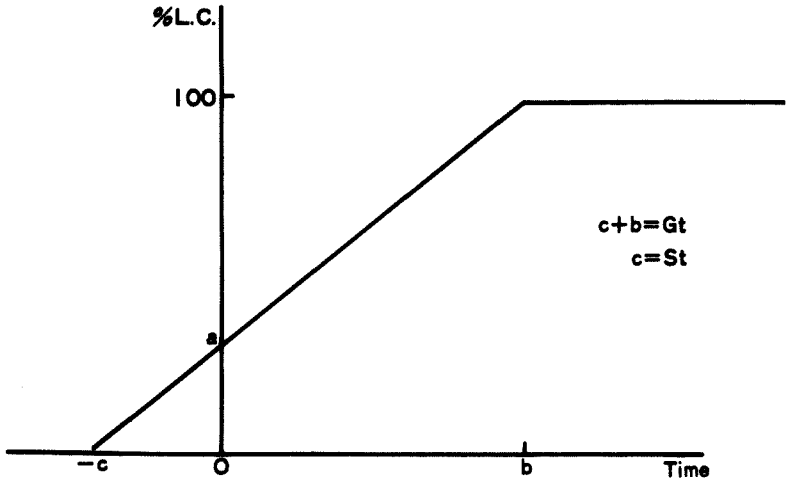

Figure 8. Analysis of cumulative labelling method.

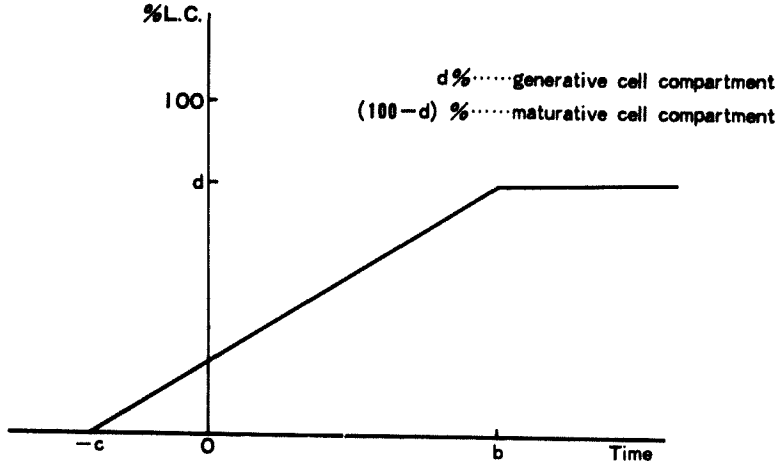

Figure 9. Cumulative labelling method and growth fraction. The value of "d" shows growth fraction.

was 2 h. Since labelled mitoses were observed 2 h after the start of H^3-Thymidine administration (Figure 11), Gt_2 was 2 h while the mitotic Index (M.I.) was 0.07. Therefore Mt was known to be about 1.6 h. Accordingly, from the equation, it was also known that Gt_1 was about 17.4 h.

Next, the P.L.C. of osteoblasts was 14 per cent at 2 h after ad-

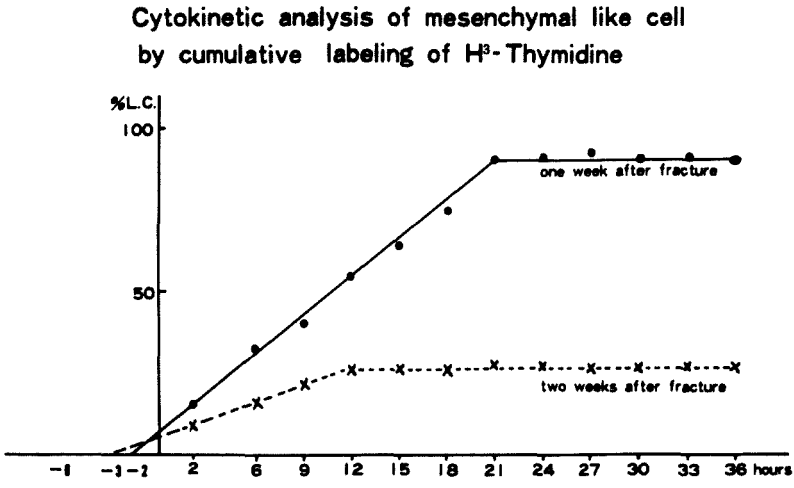

Figure 10.

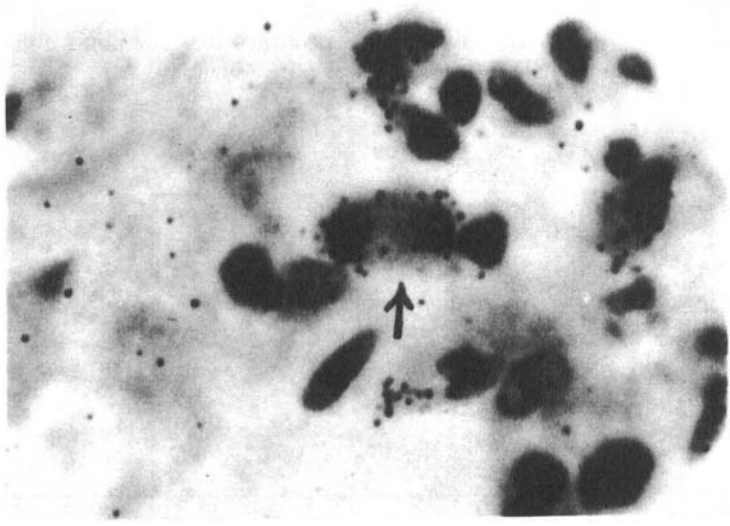

Figure 11. Labelled mitosis of mesenchymal-like cell is observed. One week after fracture and 2 h after starting the administration of H³-Thymidine. × 1000.

ministration of H³-Thymidine, and thereafter it increased almost linearly, reaching 72 per cent in 18 h. Thereafter, the curve formed a plateau as indicated in Figure 12. From Figure 12 it is known that Gt was 20 h and St was 2 h. Since the time span from the start of the administration of H³-Thymidine to the initial appearance of labelled

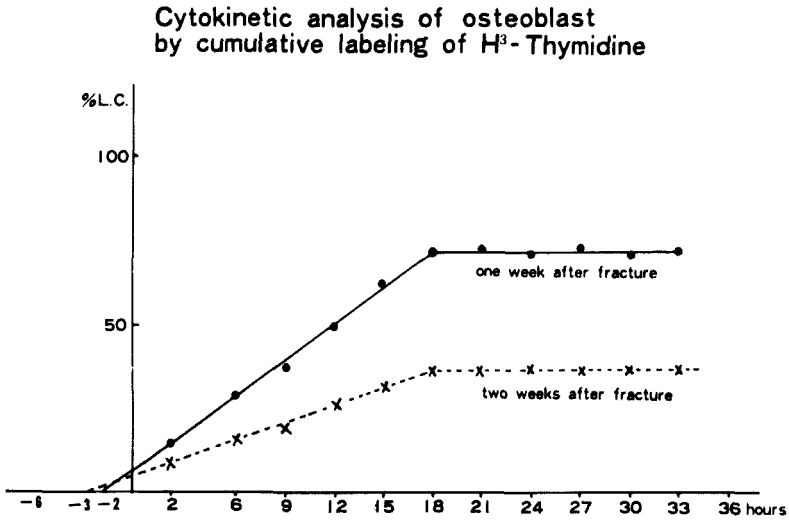

Figure 12.

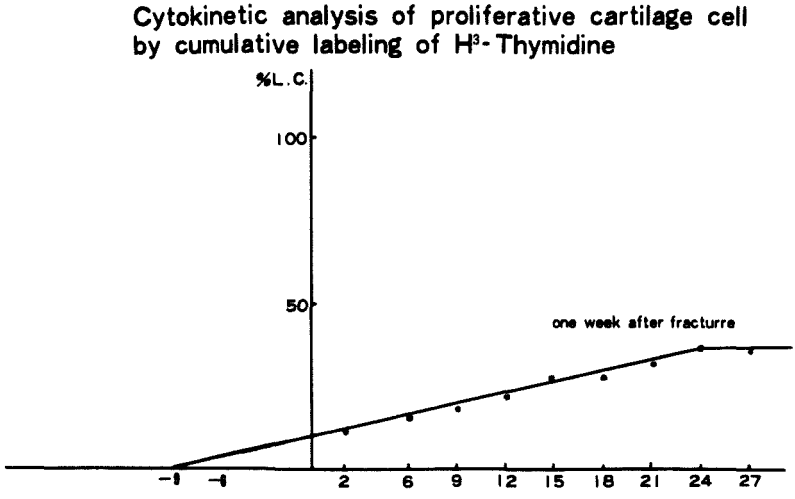

Figure 13.

mitoses was 6 h, Gt_2 was 6 h. Since M.I. was 0.02, Mt was 0.4 h. Accordingly, from the foregoing equation, Gt_1 should be 11.6 h. As for the cartilage cells, DNA synthesis was observed only with proliferative cartilage cells. These labelled proliferative cartilage cells were able to be observed adjacent to the group of labelled mesenchymal-like cells and their P.L.C. reached 12 per cent after the administration of H^3-

Thymidine and thereafter increased up to 38 per cent in 24 hours. Subsequently it remained in the range of 36–38 per cent to form a plateau (Figure 13). From Figure 13, it is known that Gt was 33 h and St was 9 h. However, since no mitosis was observed with these cells it was impossible to obtain Mt, Gt_1 and Gt_2.

Analysis 2 weeks after fracture. The P.L.C. of mesenchymal-like cells increased linearly after the start of the administration of H^3-Thymidine as indicated in Figure 10, and reached 25 per cent after 12 h. Thereafter the curve formed a plateau. From this Figure, it is known that Gt was 15 h and St was 3 h. Since labelled mitoses first appeared 2 h later, Gt_2 was thus 2 h. From M.I. (0.04) Mt was calculated to be about 0.6 h, from which Gt_1 was calculated at about 9.4 h.

The P.L.C. of osteoblasts increased linearly after the start of the administration of H^3-Thymidine as indicated in Figure 12. Eighteen hours later, it reached 31 per cent and thereafter the curve formed a plateau. From Figure 12, it is known that Gt of osteoblast 2 weeks after fracture was 21 h and St was 3 h. Since labelled mitoses first appeared at 6 h, Gt_2 was 6 h. Since M.I. was 0.02, Mt was calculated to be 0.4 h and consequently Gt_1 was 11.6 h. In the case of the proliferative cartilage cells no labelled cells were found within 9 h of the start of the administration of H^3-Thymidine but even 12 h later, P.L.C. indicated 11 per cent with no further increase. Consequently, cytokinetic analysis was impossible.

Growth fraction of the aforementioned cellular systems. When the P.L.C. curve reaches 100 per cent and then forms a plateau in the cumulative labelling of a cellular group, the growth fraction should be 1.0. If so, it is considered to be an almost uniform group of proliferative cells (Figure 8). However, as shown in Figure 9, if the P.L.C. of H^3-Thymidine reaches only up to "d per cent", it means that the amount of proliferative cells in that particular cellular group is only "d per cent", and therefore the growth fraction is $\frac{d}{100}$. Based on the above concept, it is known that for mesenchymal-like cells the growth fractions at 1 and 2 weeks after fracture were 0.9 and 0.25, respectively, while those of osteoblasts were 0.72 and 0.31, respectively. As for proliferative cartilage cells, the growth fraction 1 week after fracture was 38 per cent but that 2 weeks after was unavailable due to the reasons given above.

Time-wise change of P.L.C. after fracture (2-hour Labelling Index). On the 3rd day after fracture the proliferation of mesenchymal-like

cells was observed in the fractured part, starting at the outer periosteum region. The P.L.C. of mesenchymal-like cells at 2 h after administration of H^3-Thymidine indicated 18 per cent. On the 5th day after fracture the P.L.C. of mesenchymal-like cells indicated 16 per cent. Labelled cartilage cells were found adjacent to the region where labelled mesenchymal-like cells existed and their P.L.C. was 24 per cent. On the 7th day after fracture, formation of osteoid was observed and the percentage of labelled osteoblasts was 11 per cent while that of labelled mesenchymal-like cells was 12 per cent. Labelling of cartilage cells was also 12 per cent. In the cartilagenous tissue the grains were found only in the proliferative cartilage cells which appeared to be transformed from the mesenchymal-like cells. On the 10th day after fracture, the P.L.C. of osteoblasts was 10 per cent and that of mesenchymal-like cells was 11 per cent, but no labelling of cartilage cells was found. On the 14th day, the P.L.C. of mesenchymal-like cells was 9 per cent and that of osteoblasts was 8 per cent. DNA synthesis was observed for both of these cells but no labelling was seen in the cartilage cells.

DISCUSSION

In 1962, Bassett (1962) proposed the concept of the differentiation of pluripotential cells in connection with the genesis of osteogenic and chondrogenic cells and proved that there existed the process of mesoderm → primitive fibroblast → milieu → cartilage (chondroblast), bone (osteoblast) and fibrous tissue (collagenoblast) by *in vitro* experiments using a chick embryo tibia. Urist et al. (1965) said that mesenchymal-like cells might form cartilage or fibrous or osteoid tissue according to the milieu and that the environmental condition was one of the key elements which determined the direction of differentiation. Tonna & Cronkite (1961) administered H^3-Thymidine to Swiss Albino mice and observed the change of the prognosis of a fractured femur in terms of Labelling Index. As a result, they found that in the fractured region, proliferation of mesenchymal-like cells was quite strong and originated from the periosteum. These experiments were intended to prove that mesenchymal-like cells would differentiate into osteoblasts under certain conditions and stimuli.

However, no detailed examinations have so far been made in regard to the cytokinetics of osteogenic cells in the process of recovery after fracture. Nor have there been any reports on experiments to indicate

Table 1. Cell cycle and growth fraction of mesenchymal-like cell and osteoblast at 1 and 2 weeks after bone fracture.

		Gt	St	Mt	Gt_1	Gt_2	Growth fraction
Mesenchymal-like cell	One week after fracture	23 h	2	1.6	17.4	2	0.9
	Two weeks after fracture	15 h	3	0.6	9.4	2	0.25
Osteoblast	One week after fracture	20 h	2	0.4	11.6	6	0.72
	Two weeks after fracture	21 h	3	0.4	11.6	6	0.31

the rate of transformation into G_0 period cells and functional cells (F cell) in the cell cycle of osteogenic cells.

In this experiment, the first result obtained was that mesenchymal-like cells and osteoblasts had their own cell cycles but that these cycles might change in the stages after the fracture. Mesenchymal-like cells in particular indicated the remarkable changes of such cycles. Generation time one week after fracture was 23 h but 2 weeks later it shortened to 15 h and such shortening was mainly caused by shortening of the duration of Gt_1. On the other hand St indicated a slight prolongation (Table 1). This shortening of Gt was completely unexpected because Gt had been estimated to be shorter in the earlier period when cell proliferation was more vigorous. As to osteoblasts, unlike the case of mesenchymal-like cells, slight prolongation of Gt and St was observed 2 weeks after fracture, as indicated in Table 1. What was common in these two cell systems was the finding that the angle of slope of the upward going line of the P.L.C. indicated in Figures 10 and 12 became smaller 2 weeks after fracture. Such a reduction of the angle of slope means a prolongation of St and a decrease of growth fractions.

The second finding obtained in this experiment was the change of the growth fractions. In the case of mesenchymal-like cells the growth fraction changed from 0.9 to 0.25 in the period from 1 to 2 weeks after fracture, and a radical decrease of the generative cell compartment was observed. Seventy-five per cent of these mesenchymal-like cells were considered as the functional cells and G_0 period cells. The former will

differentiate, as stated later, in two directions, namely osteoblast and cartilage cell, whereas the latter is thought to transform into the resting mesenchymal-like cells. The same mechanism is likely with osteoblasts and in the light of the change of growth fraction from 0.72 to 0.31 it may be possible that there are cell populations which transform into osteocyte and resting non-dividing osteoblasts. From these facts, it is supposed that each cell system controls the decrease of its own generative cell compartment depending upon the degree of requirement of the reintegration of a fracture.

The third interesting finding was the uptake test with S^{35} and H^3-Proline. This test revealed that mesenchymal-like cells had the potential to develop into osteoblasts and cartilage cells. The mesenchymal-like cells indicating transition into osteoblasts (osteoid) had a greater uptake of H^3-Proline while those indicating transition into cartilage cells (cartilage tissue) had a greater uptake of S^{35}. This fact suggests that mesenchymal-like cells potentially have two functional natures and they are pluripotential in that they can differentiate into either osteoblasts or chondroblasts. The osteoblasts which were differentiated from mesenchymal-like cells indicated vigorous intracellular H^3-Proline uptake, and in a short time (within 2 h) grains of H^3-Proline were discharged into the matrix of the osteoid. As stated above, osteoblasts are evidently the functional cells, but osteoblasts are not only a result of cellular flux from mesenchymal-like cells but also function as generative cells in the succeeding system from osteoblasts to osteocytes. On the other hand, cartilage cells indicated strong uptake of S^{35}, but they discharged S^{35} into the matrix of the cartilage tissue in a short time and thus fulfilled an important role in the formation of the matrix of the cartilage. However, labelling of H^3-Thymidine started from proliferative cartilage cells only, and DNA synthesis of cartilage cells occurred only in the region adjacent to mesenchymal-like cells showing high P.L.C. At one week after fracture, the generation time of proliferative cartilage cells was 33 h and St was 9 h, which were longer than those of their cell systems. In addition, their growth fraction was 0.38 which was lower than that of other cell systems. From these facts it may be concluded that 1 week after fracture, this cell system is not positively concerned with the reintegration of the fracture. The fact that no increase of P.L.C. was observed 2 weeks after fracture would support the above conclusion.

The findings obtained on the 3rd, 5th, 7th, 10th and 14th days after fracture in regard to H^3-Thymidine uptake of various cell systems 2 h

after administration suggest that proliferation of mesenchymal-like cells occurs first and then follows the process of mesenchymal-like cells → cartilage cells followed by the process of mesenchymal-like cells → osteoblasts.

The last but quite interesting point is whether or not there is the process of transformation from chondrocytes to osteoblasts. According to Bently & Greer (1970), it is said that chondrocyte in epiphyseal plate is not the precursor of osteoblast in the process of enchondral ossification. In the present experiment:

(1) No labelling was noticed in degenerative cartilage cells during the 36 h period of observation after cumulative labelling of H^3-Thymidine.
(2) Increase of P.L.C. of osteoblasts was linear and did not result in double-phase lines having an upward inclination on a graph.
(3) The two cell systems of osteoblasts and cartilage cells were functionally different.

From the above three findings, it was considered that the process of transformation from chondrocytes to osteoblasts was unlikely to occur at cellular level but on the other hand it was proven that in spite of no intracellular uptake of S^{35} in osteoblast, the grains were observed in the matrix of the osteoid and if so, cartilage tissue would be significant in the mechanism of ossification.

CONCLUSION

As the result of cytokinetic analysis of osteogenic cell at the time of reintegration after experimentally administered bone fractures in mice, the following findings were obtained:

1) Gt of mesenchymal-like cells 1 week after fracture was 23 h but that 2 weeks later was 15 h. This shortening of Gt was due to the shortening of Gt_1. Gt of osteoblasts, one week after, was 20 h and that 2 weeks after was 21 h. This prolongation of Gt was due to the prolongation of St. Despite the fact that Gt of mesenchymal-like cells 2 weeks after fracture was shortened, St indicated prolongation.
2) Both mesenchymal-like cells and osteoblasts indicated a reduction of growth fractions 2 weeks later. From these facts, it is considered that in the reintegration of the fracture, these proliferative cells

control their own growth fractions according to the necessity of repair of the fractured part.

3) It was argued that chondrocytes originated from mesenchymal-like cells but should not be the precursors of osteoblasts.
4) It was shown that mesenchymal-like cells must have the potential to differentiate into either osteoblasts or chondrocytes.
5) Mesenchymal-like cells are the precursors of osteoblasts and the latter are the precursors of osteocytes, but mesenchymal-like cells and osteoblasts have their own respective cell cycles.
6) It was argued that the relation between S^{35} uptake by the matrix of the osteoid and chondrocytes as functional cells should be studied in relation to the role played by cartilage tissue in the mechanism of ossification.

REFERENCES

Bassett, C. A. L. (1962) Current concepts of bone formation. *J. Bone Jt Surg.* **44**-A, 1217–1244.

Bentley, G. & Greer, R. B. (1970) The fate of chondrocyte in endochondral ossification in the rabbit. *J. Bone Jt Surg.* **52**-B, 571–577.

Kember, N. F. (1971) Cell population kinetics of bone growth. *Clin. Orthop.* **76**, 213–230.

Tonna, E. A. (1958) Histologic and histochemical studies on the periosteum of male and female rats at different age. *J. Geront.* **13**, 14–19.

Tonna, E. A. & Cronkite, E. P. (1961) Cellular response to fracture studied with tritiated thymidine. *J. Bone Jt Surg.* **43**-A, 352–362.

Tonna, E. A. & Cronkite, E. P. (1962) An autoradiographic study of periosteal cell proliferation with tritiated thymidine. *Lab. Invest.* **11**, 455–562.

Urist, M. R., Wallace, T. H. & Adams, T. (1965) The function of fibrocartilaginous callus. *J. Bone Jt Surg.* **47**-B, 304–318.

Walker, K. V. R. & Kember, N. F. (1972 a) Cell kinetics of growth cartilage in the rat tibia. I. Measurements in male rats. *Cell Tiss. Kinet.* **5**, 401–408.

Walker, K. V. R. & Kember, N. F. (1972 b) Cell kinetics of growth cartilage in the rat tibia. II. Measurements during ageing. *Cell Tiss. Kinet.* **5**, 409–419.

Key words: cytokinetic; osteogenic; fracture

Correspondence to:

Dr. Shohei Manabe
Department of Orthopaedic Surgery
Kanazawa University Medical School
Kanazawa City, Japan