endomysial and perimysial connective tissue between fibres and fasciculi was noted in the ipsilateral EDL as previously reported by other investigators for the hypertrophic soleus muscle[20].

The finding that there was neither significant loss nor recruitment of myonuclei into hypertrophied muscle appears to suggest that true splitting of muscle fibres does not occur. If fibre splitting had actually occurred, nuclei of muscle fibres would have been lost to satellite structures or the smaller 'split' fibres, and then the fall in hypertrophic N_V values would have been much greater. The electron microscopic findings suggest that satellite structures take origin from satellite cells rather than by any splitting from muscle fibres. The failure to find ^3H tritium labelled nuclei in hypertrophic fibres would also seem to suggest that either fusion of satellite cell products with muscle fibres does not take place or that if it does occur extensive mitosis within satellite structures does not occur prior to fusion. Refusion of split fibres with their parent fibres or fusion of satellite structures with mature fibres would seem to be prohibited by the occurrence of a prominent endomysial sheath clearly demonstrable using picro sirius blue GL[12].

These findings lead us to believe that the transient histological features of hypertrophying muscles could be regarded as a reaction to the hypertrophy-inducing process rather than an essential feature of the mechanism of fibre size increase. The findings lead us to believe that split fibres or satellite structures either arise by satellite cell division but subsequently undergo complete regression or that they arise from satellite cell activity without significant mitosis and subsequently fuse with the previously existing fibres. These conclusions are at variance with the traditionally accepted views and remain to be tested experimentally.

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An improved method for calculating colony forming ability in soft agar with special reference to malignancy

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Summary. We examined the correlation between the tumorigenicity and the growth capacity in soft agar of various malignant cell lines. The colony forming rates were calculated in (a) 0%, (b) 0.15%, (c) 0.30%, and (d) 0.40% soft agar medium. An approximate value for the colony forming capacity, 'y', was then established for the formula $y=\frac{1}{a}\int_0^a f(x)\,dx$ (x = concentration of soft agar, f(x) = rate of appearance of colony forming cells with x% soft agar medium).

Malignancy in cultured cell lines is indicated by the properties the cell lines show in vitro or in vivo such as a) loss of contact inhibition[2], b) growth in soft agar[3], c) growth in spinner culture[4], and d) tumorigenicity[5]. The most reliable method of determining the degree of malignancy would be to examine the tumorigenicity of each cultured cell line, but it is not possible in a short-term experiment to do a quantitative study. In order to study the mechanism by which malignancy is controlled, a quantitative study of the cell variation seen in malignant cells is needed. In this experiment we introduced a new method of calculating the colony forming ability, and investigated whether or not cell growth in soft agar correlates with tumorigenicity, and whether or not the former could be applied to the quantitative study of malignancy in vitro.

Materials and methods. The cell lines used were Ht(A/Jax mouse), F(A/Jax), MC(A/Jax), m(A/Jax), L(C_3H), MRCB(A/Jax), SY(A/Jax), and ME(A/Jax) as well as normal mouse embryonic cells (MEb) and normal human embryonic lung cells (HEL).

Tumorigenicity: The tumorigenicity of the various cell lines which we used was tested by injecting 1×10^6 cells into the subcutaneum of an isologous strain of newborn, adult and nude mice(BALB/c, nu/nu). The latency period (the length of time until tumors were palpable in the site at which the cells were injected) and the days of survival of 50% of the mice into which the cells were injected, were noted.

Cell growth in soft agar: 5000 cells were seeded in a petri dish and their colony forming ability was examined on the 5th day after cell seeding. Any group of cells consisting of more than 6 cells derived from a single cells was classified as a colony. We ascertained that the cell lines which we used, except for normal cell (MEb and HEL), ME and SY cell lines, by the 5th day had produced colonies large enough to be identified under the microscope, and that the colony forming cells had divided an average of 2.5 times within a period of 5 days. Normal cell (MEb and HEL), ME and SY cell lines were observed for about 1 month in order to determine whether or not they had colony forming ability. For each group of 3 dishes, the mean value of the numbers of colonies found in them was calculated and the colony forming rates were obtained.

Preparation of bottom agar layer: 6 ml of 0.6% agar growth medium were poured into 60-mm petri dishes and allowed

to harden just before using. This agar layer was produced by mixing an equal part of 2× growth medium brought to 37 °C and an equal volume of agar in a 1.2% solution with distilled water.

Plating of cells in top agar: The cells were treated with 0.25% trypsin for detachment of cells, for 5 min, and then a few ml of growth medium was added. In this experiment, cells were used before the cell density in the culture vessels reached confluency. Otherwise, cell clumps and cell death could not be avoided. The 6 ml of growth medium which contained the cells, and in which agar had been dissolved at concentrations of 0, 0.15, 0.30, and 0.40%, was gently poured over the hardened bottom layer.

Colony forming ability: Colony forming ability in various cell lines can be expressed in the following formula, when the concentration of soft agar in growth medium changes from zero to any other percentage.

$$y=\frac{1}{a}\int_0^a f(x)\,dx \quad \ldots\ldots (1)$$

Here, y = colony forming rate. x = concentration of soft agar. f(x) = rate of appearance of colony forming cells at x% soft agar in growth medium. Practically speaking, it is very difficult to establish the function f(x). Therefore, we must be satisfied with calculating the area below f(x) by approximation in order to calculate $\int_0^a f(x)\,dx$. The simplest method is:

$$\int_0^a f(x)\,dx=\frac{1}{2}\cdot\frac{a}{n}\left[\left\{f(0)+f\left(\frac{a}{n}\right)\right\}+\left\{f\left(\frac{a}{n}\right)+f\left(2\cdot\frac{a}{n}\right)\right\}+\cdots+\left\{f\left(\overline{n-1}\cdot\frac{a}{n}\right)+f\left(n\cdot\frac{a}{n}\right)\right\}\right]\cdots \quad (2)$$

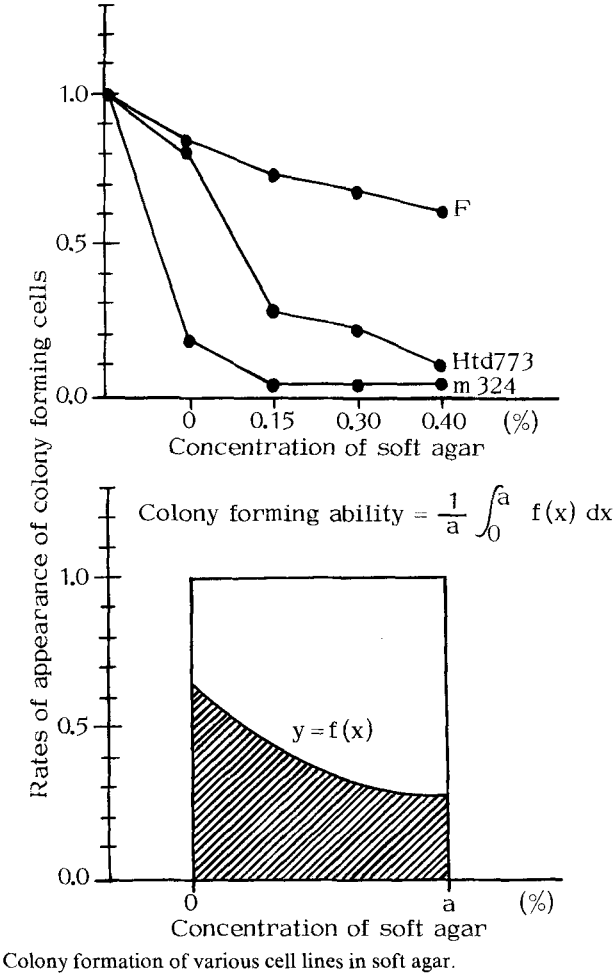
In the figure, the relationship between colony forming rates and colony forming ability is shown. This shows the decrease in colony forming rates accompanying the increase in the concentration of agar.

Results. Tumorigenicity: The results of transplantation are summarized in the table. Htc1-4 and m cell lines showed a low degree of malignancy. The degree of malignancy of m cells in the 324th generation was found to have decreased when compared to that of the 104th generation. The cells of the 324th generation showed no tumorigenicity even when they were injected into an isologous strain of newborn mice. The cells of the L and MRCB cell lines were able to produce tumors in newborn mice of an isologous strain, but did not show tumorigenicity in adult mice. The cells of Htd773, F, MC, ME, and SY were able to kill the hosts into which they were injected. The HEL (human embryo lung cells) and MEb (mouse embryo cells) were normal cells.

Colony forming ability: The values representing colony forming ability as calculated in formula (1) for each cell line, are listed in the right hand column of the table. The values of the colony forming ability in m, Htc1-4, MRCB and Htd773 cell lines which showed a low degree of malignancy in transplantation experiments, were lower than those of highly malignant F and MC cell lines but higher than those of normal cells. The sizes of the colonies produced are listed in the table. They are represented by the average values of the largest 30 colonies in each of 30 visual fields in 0.3% agar medium. Highly malignant cell lines such as F and MC, showed a tendency to produce larger colonies than the cell lines having a low degree of malignancy. This suggests that there may be a difference in the number of factors controlling colony forming ability which may also influence the difference in the size of a colony. However, the L cell line is an exception. With ME and SY cell lines which had no ability to produce colonies in soft agar culture, we experimented with culture media other than TC199. With none of these culture media could we obtain positive results.

Colony forming ability, doubling time, and saturation density: As shown in the table, there was no correlation between the colony forming ability and the doubling time of any cell line. The doubling time of m and L cell lines are 21 and 22 h respectively. These times are shorter than those of MC and F cell lines. However, the saturation density shown by each cell line correlated with its colony forming ability. The saturation densities of m and Htc1-4 cell lines with low colony forming ability values, were lower than those of F, MC, and Htd773 cells which have high colony forming ability values. ME and SY cell lines which have no capacity to produce colonies in soft agar, showed an even lower degree of saturation density.

Discussion. This experiment showed that the greater the colony forming ability was, the higher the degree of malignancy would be. However, as shown by ME and SY cell lines, there was a contradiction in that they demonstrated no colony forming ability even though they were able to produce tumors in an isologous strain of A/Jax mice. The reason for this result was not clear, but although we used different kinds of media (Eagle, RPMI, 199 and Ham) with 10% serum (calf or fetal calf serum) and observed the growth of these cell lines for more than 1 month, they



Colony formation of various cell lines in soft agar.

Tumorigenicity, doubling time, saturation density and colony forming ability

Cell line	Tumorigenicity Nude	Newborn	Adult	Rates of tumor production	Latency period (days)	50% survivals (days)	Doubling time (h)	Saturation density ($\times 10^4$/cm^2)	Sizes of colonies radii (r) (arbitrary units)	Colony forming ability
MEb	–	–	–	–	–	–	96	1.2	0	0
HEL	–	–	–	–	–	–	108	2.3	0	0
Htc1-4	–	–	–	–	–	–	50	26.9	–	0.11
m 324	+	–	–	3/3***	47	106	21	38.9	0.33±0.08	0.05
m 104	+	±	–	10/13**	7	42	21	36.0	0.44±0.04	0.05
MRCB	+	±	–	7/11**	10	33	19	36.7	1.47±0.56	0.07
L	+	±	–	6/11**	9	32	22	42.0	0.62±0.08	0.52
Htd773ca	+	+	+	5/5*	82	120	–	–	–	0.07
Htd773	+	+	+	8/8*	23	52	24	45.7	0.64±0.13	0.36
F	+	+	+	4/4*	21	70	36	55.1	1.29±0.33	0.65
Fsf14MEM	+	+	+	5/5*	28	86	35	56.0	–	0.75
MC	+	+	+	4/4*	6	70	22	75.5	1.10±0.18	0.85
ME	+	±	±	3/5*	99	140	24	18.5	–	0
SY	+	+	+	11/11*	20	90	22	15.0	–	0

*** Nude, ** newborn and * adult mice.

showed no capacity to produce colonies. It is known that various factors, such as polyanionic substances in agar, collagen, serum levels, insulin, serin and other amino acids, influence the colony forming ability in a suspension culture of untransformed cells[4]. However, Sanders et al. reported that some of these factors have no relationship to the growth of transformed cells in soft agar[6]. Our SY and ME cell lines have only been maintained for less than a year after the cells had spontaneously transformed, and this is less time than other cell lines which we have been maintaining in vitro for more than 10 years. The SY and ME may not have adapted to culture conditions enough to produce colonies. We are now investigating these possibilities. The advantages of calculating colony forming ability with formulas (1) or (2) were that a) we could clearly determine the various degrees of malignancy of the cells which showed no growth in 0.3% or 0.4% soft agar, and b) we could represent the degree of malignancy as a single value, even though different values representing colony forming rates were obtained at different concentrations of agar. We could calculate the colony forming ability easily if a general function 'f(x)' could be established. Hitherto, the cells which could grow in a soft solid agar have been considered to be malignant. On the other hand, it is well-known that many cell lines, although not normal, show neither tumorigenicity nor growth in soft agar medium at these concentrations. In our method, we were able to differentiate those which were neither malignant nor normal from those which were either malginant or normal, and allocate them a position between those of normal and malignant cell lines. It could open new possibilities for the elucidation of the mechanism of the control of malignancy if cell lines having different degrees of malignancy could be compared on the same table. Finally, we would like to mention those problems which should be taken into consideration in a soft agar experiment. The colony forming ability is greatly influenced by a) the state of the cells used, b) the period of trypsin treatment, c) the temperature of the agar medium at the time when the cells were planted, and d) the lot of serum used. These 4 points should be watched with care in every experiment in which an agar medium is used. In order to elucidate the genetic control of malignancy, first of all, we must determine the phenotype of malignancy and do a quantitative study of this phenotype. In this experiment, we tried to find out whether or not growth in soft agar correlated with the degree of malignancy shown in vivo, and explore the possibility of applying this method to the quantitative study of malignancy. This experiment produced satisfactory results in this regard.

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Effect of 2-mercaptopropionylglycine (MPG) on thyroid function in sub-lethally irradiated mice[1]

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Summary. External irradiation resulted in an increase in thyroid ^{131}I uptake and plasma PB ^{131}I conversion ratio, whereas pretreatment with MPG reduced both the values significantly. Metabolic inhibition is suggested as a possible mechanism of action by the drug.

Previous studies in our laboratory have demonstrated the radioprotective effect of MPG on the different tissues which are directly responsible for the radiation death in mice[2-5]. Since thyroid plays an important role in determining the basal metabolic rate and since the metabolic state influences radiosensitivity, the present study has been carried out to determine the effect of MPG on thyroid function in mice exposed to 500 r of ^{60}Co gamma radiation.

Material and method. 40 adult male Swiss albino mice were selected from an inbred colony and divided into 2 equal groups, control (+500 r) and experimental (+500 r +MPG). The animals were treated as described earlier[3]