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EFFECTS OF LOW ENERGY BETA - IRRADIATION FROM TRITIATED WATER ON THE MORPHOLOGY OF 3T3 FIBROBLASTS

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Abstract

Cellular alterations of cultured 3T3 cells irradiated with beta-rays from tritiated water were studied by scanning and transmission electron microscopy. We observed decreased negative surface charges, vacuolization of rough endoplasmic reticulum and Golgi-complex, degeneration of mitochondria, increase of lysosomal activity and changes in distribution and amount of microfilaments in the irradiated cells, that parallelled changes in cell shape.

Key words: beta irradiation, tritiated water, 3T3 cells, morphology, cationized ferritin

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Introduction

A considerable amount of tritium, as tritiated water, is produced by nuclear power plants already under normal conditions, and the β-radiation might be significant if accidents occur (2, 17). Tritiated water (HTO) can easily enter the living organism by inhalation, ingestion and/or through body surfaces and be incorporated in different cellular structures (41). Most of the HTO is localized in the water surrounding the biological macromolecules (40, 51). There are many experimental results about toxicity carcinogenic and mutagenic action of HTO (4, 5, 11, 12, 15, 24, 25, 35, 37, 38, 41, 47, 48, 50, 59, 65, 67) and on the histological changes of cells (4, 5, 8, 15, 26, 27, 32, 38, 41, 44) e.g., hepatocytes, or oocytes following its application. HTO induces the formation of micronuclei in bone marrow cells of mice maintained on 15 and 30 μCi/ml (27). Decrease of ovarian volume, fragmentation of the nuclei of oocytes, pseudomaturation and spindle formation following HTO treatment were observed (26). The granulose cells of the ovary also showed pyknosis and cell lysis (26). Atrophy after HTO treatment was observed in the spleen and thymus (59). Recently, cell death via apoptosis was demonstrated in mouse intestine after continuous irradiation with β-rays from tritiated water (22, 23).

Fewer data are available on the membrane effects of HTO. Our previous work describing changes of lectin binding capacity in cultured cells (30), and observations on immunological changes of cells (28, 64, 69) also demonstrated membrane alterations after HTO treatment. Therefore, the question was raised whether β-irradiation from tritiated water causes alterations in the ultrastructure of cells and in the supramolecular organization of their plasma membrane similar to those observed following X- or gamma-irradiation (6, 14, 30, 31, 58, 62). Data presented here demonstrate the injurious effect of tritiated water (β-irradiation) on the structure of 3T3 fibroblasts.

Materials and Methods

Cell culture

Mouse embryo 3T3 fibroblasts were cultured in

Eagle MEM medium supplemented with glutamine (4 mM final concentration), 10% fetal calf serum. They were maintained at 37°C in a humidified 5% CO_2 - 95% air atmosphere. The cells were used in semiconfluent monolayers.

Irradiation

Exponentially growing 3T3 cell were incubated with various concentrations of tritiated water (0.37, 3.7, 37, and 370 kBq/ml) for 1, 3, 6 and 24 hours. The absorbed dose was calculated according to Liber et al. (35), i.e., 37 kBq/ml HTO is equivalent to 2.3 mGy/24 hours, or 1.6 μGy/min.

Cytochemistry

Negatively charged sites were visualized by cationized ferritin binding (9). Prior to ferritin binding the cells were fixed for 30 minutes in 0.1 M phosphate buffered 0.025% glutaraldehyde (pH 7.3).

Transmission and scanning electron microscopy

The cells were fixed for 1 hour in 0.1 M phosphate buffered 2.5% glutaraldehyde (pH 7,3, 4°C), postfixed in 1 per cent OsO_4, dehydrated with alcohol or acetone, and embedded in Durcupan AC (Fluka). Ultrathin sections were cut with glass or diamond knives on an LKB ultramicrotome. The sections were examined by a TESLA transmission electron microscope (TEM).

For scanning electron microscopy (SEM), the samples were dehydrated, and dried in a Sorvall critical point drying apparatus and coated with gold. Specimens were viewed and photographed using a JSM 50A SEM operating at an accelerating voltage of 20 kV, and a tilt angle of 45°.

Results

Micromorphological alterations

The polygonal cells of normal semi-confluent 3T3 mouse fibroblasts cultures are typically flat. They have a few short microvilli and their lamellipodia are partly lifted from the substrate. The cells and their edges usually adhere to the substrate or are attached to other cells. Thin, long cytoplasmic extensions (filopodia) are centripetally oriented from the free surfaces (Figs. 1 and 2). Under the different experimental conditions used in this study, changes of cell shapes were caused only by the 37 kBq or more HTO applied for 24 hours. After this treatment, the cell substrate and cell-cell contacts were loosened or lost and the cell edges elevated from the substrate. Some cells exhibited elongated or rounded forms (Figs. 3 and 4). The colliding cells often passed over each other (Fig. 3, inset). The control culture contains about 3-5% non-fibroblastic form of cells, the HTO treated ones approximately 60-70%.

Fine structural alterations

The nucleus of untreated cells has an ellipsoidal form with a few small invaginations on its surface and contains fairly uniformly dispersed chromatin (Fig. 5a). The cell has a few cisternae of rough endoplasmic reticulum (RER) (Figs. 5-7). The numerous free ribosomes are scattered throughout the cytoplasm. The well-organized Golgi complex is localized near the nucleus (Fig. 5). The microfilaments are randomly dispersed in the cytoplasm and form a cortical network under the plasma membrane (Figs. 6 and 7) and in the villi. Mitochondria have a typical cylindrical shape with well-packed and organized cristae (Figs. 6 and 7).

Following HTO treatment (37 kBq/ml) deep invaginations and cytoplasmic indentations appeared on the surface of the nuclei (Figs. 8 and 9). The chromatin has transformed into the condensed state. Vacuolization of the RER and swelling of mitochondria were frequently observed (Figs. 9 and 10). The Golgi complex was fragmented and an increased number of Golgi vacuoles could be seen in the perinuclear region (Fig. 10). The cells contained large numbers of lysosome-like bodies, lipid vacuoles, and vacuoles filled with partially digested cell organelles (Figs. 8, 10).

Alteration of negatively charged sites

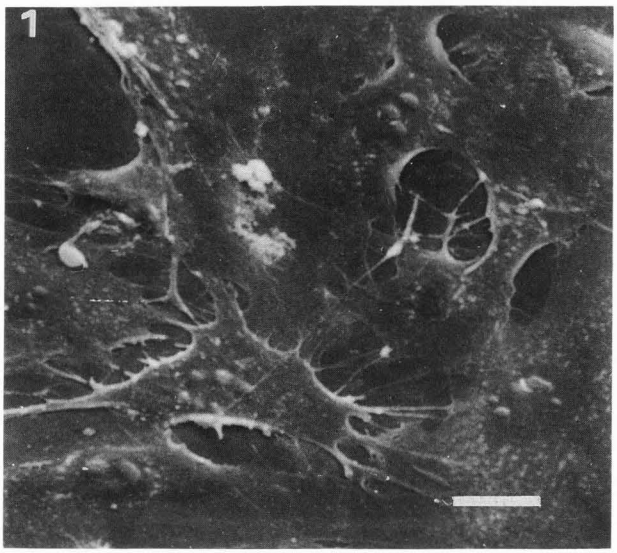
A continuous layer of cationized ferritin, a marker of distribution of negative charges was bound to the apical part of the untreated cells (Fig. 11a). Similar to effects of other kinds of ionizing radiations (31, 58), HTO treatment decreased the cationized ferritin binding capacity of the plasma membrane and the ferritin particles were distributed on the cell surface in clusters (Fig. 11b).

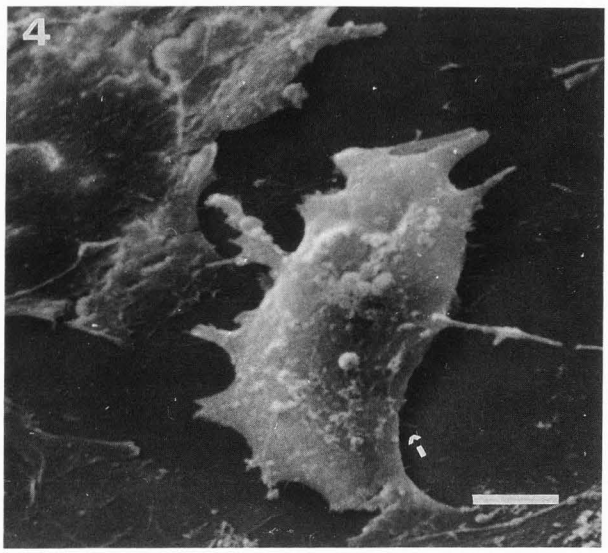
Discussion

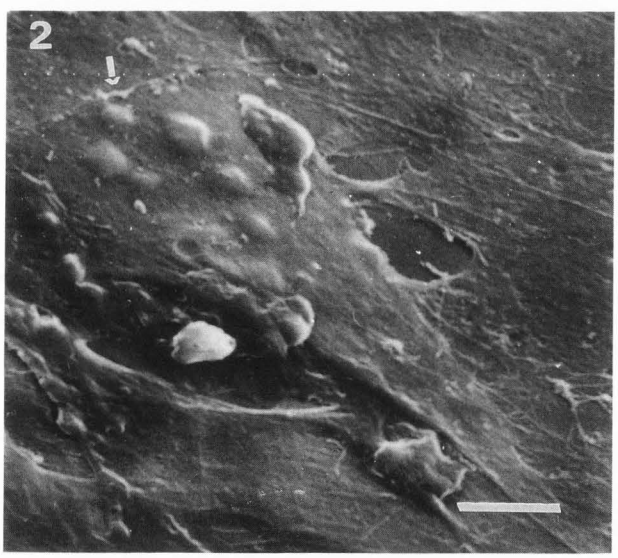
Our results show that the β-irradiation from tritiated water induces various morphological changes including effects on membrane. The observed alterations, however, are not specific. Changes of cell shape and micromorphology of cell surface were seen following X- or gamma-radiation (20, 57, 58), and have also been described as a consequence of non-ionizing radiation or heat treatment (19, 21, 40, 68) as well as treatment with membrane-active agents (3, 34, 56). The exact mechanism is not known. There are assumptions that the decrease of negative surface charges indicates a profound perturbation of membrane structure and, consequently, function (31, 58) as is known after X-irradiation or heat treatment (31, 49).

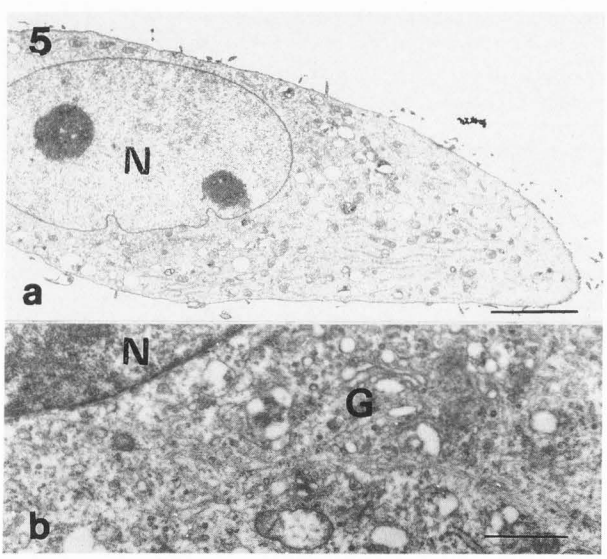
The effects of HTO are manifested in the changes of the fine structure of both nucleus and cytoplasm. Some elements of these ultrastructural changes are similar to the effects of other kinds of irradiation (1, 6, 10, 13, 29, 36, 42, 44, 54, 55-59, 61, 62).

The mechanisms of HTO induced changes of membranes are considered to be similar to those induced by other kinds of ionizing radiations (14, 39, 43, 46, 60, 66). The β-irradiation also generate oxygen free radicals and H_2O_2 both in extracellular HTO solution and in water pools of cells which contribute to the development of morphological changes of cells. It is known that the exogenously added H_2O_2 acts upon membrane integrity causing an increased membrane blebbing and changes of membrane potential (6, 18, 62, 63). The lipid peroxida-









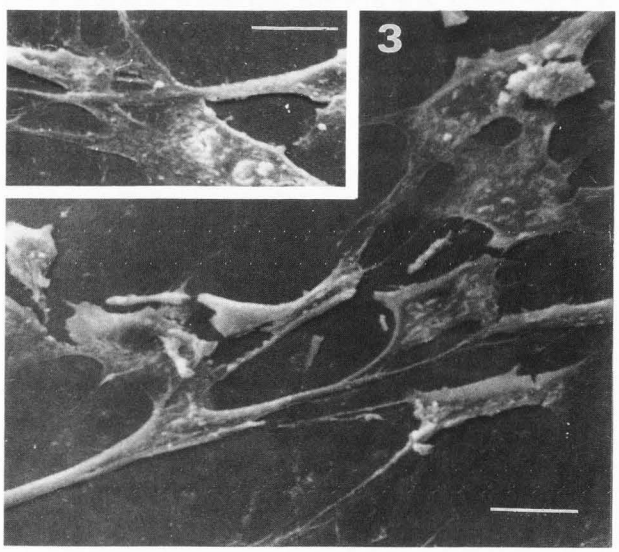


Figure 1. Overview of the monolayer of control 3T3 fibroblasts. The flat polygonal cells tightly adhere to the substrate. They have a few short villi, surface blebs are absent. Bar = 2 μm.

Figure 2. Well adhered control 3T3 cells. The arrow points to the site of cell-cell contacts. Bar = 1 μm.

Figure 3. 3T3 cells after HTO treatment (24 hours, 37 kBq/ml). The cells are separated from each other and their edges detached from the substrate. Some of the cells are spindle-shaped and rounded. The colliding cells often pass (inset). Bar (for figure and inset) = 2 μm.

Figure 4. Bleblike protrusions on rounded cells attached to the substrate via cytoplasmic bridges (-->). HTO treatment (24 hours, 37 kBq/ml). Bar = 1 μm.

Figure 5. Electron micrograph of control 3T3 cells. N = nucleus, G = Golgi complex. Bars = 1 μm (a); 0.2 μm (b).

tion products also play a role in membrane effects of X- or β-rays (39, 43, 46, 60).

At the same time, it is remarkable that these morphological changes after HTO treatment were experienced following a very low absorbed dose (2.3 mGy), while X-irradiation with 50 rad (cca. 490 mGy) is required to induce such changes in shape or fine structure in lymphocytes (7). It is known from the literature, too, that in the case of cytogenetic effects or mutations the RBE value of HTO is approximately 1 - 4.8 (11, 12, 59). Recently, biological alterations were found following treatment of mice with HTO of similar low concentrations, (e.g., in the range of 10-100 kBq (5)).

Experimental data show an inhomogeneous distribution of HTO cells (40, 41). It is localized firstly in the different water pools of cell and cell organelles (40, 41). Since the membrane bound water, which is very important to the membrane's functions and structural organization (45), contains HTO in relatively high concentration, the emitted β-particles can produce direct ionization of membranes macromolecules, too.

Nevertheless, we cannot ignore the possibility of a direct effect of tritium on membrane structure, via isotope effect, as this isotope is three times heavier than hydrogen. In fact, also deuterium exerts cytogenetic toxicity and alters the cell shape (25, 33, 70).

In summary, we suggest that the HTO causes membrane effects most probably through its β-radiation as demonstrated by morphological methods. The observation of membrane changes after treatment with low doses points to the necessity of further investigations on various biological effects of HTO.

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Figure 6. Part of the cytoplasm of control 3T3 cell. Mitochondria (M), cytoplasmic vesicles (V), cisternae of endoplasmic reticulum, microtubules (*) and microfilaments (f) and numerous polyribosomes are seen. Bar = 0.3 μm.

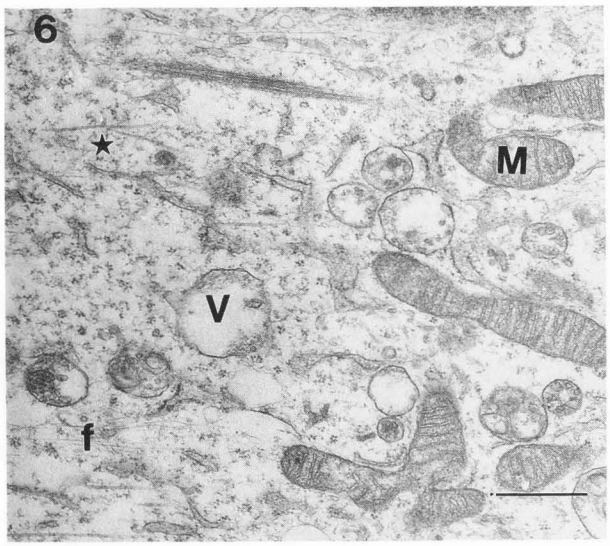
Figure 7. Detail of the perinuclear area of control 3T3 cell. Mitochondria (M), rough endoplasmic reticulum (RER), vacuole (V), nucleus (N). Bar = 0.3 μm.

Figure 8. Electron micrograph of HTO treated (24 hours, 37 kBq/ml) cells. Cytoplasmic vacuolization and extrusions of the cell surface are prominent. The nucleus (N) shows deep cytoplasmic indentations (*). Bar = 1 μm.

Figure 9. Invaginations of nuclear membrane (*). HTO treated (37 kBq/ml, 24 hours). Bar = 0.3 μm; bar for inset = 0.2 μm.

Figure 10. Altered, swollen mitochondria (M) and vacuolized Golgi complex (G) in a fibroblast treated for 24 hours with HTO 37 kBq/ml. Bar = 0.3 μm, bar for inset = 0.2 μm.

Figure 11. Binding of cationized ferritin (CF) on the surface of control (**a**) and HTO (37 kBq/ml, 24 hours) treated (**b**) specimens. CF particles are distributed in clusters after the HTO treatment, and the amount of CF bound to the surface of the cell is decreased. Bar = 0.1 μm.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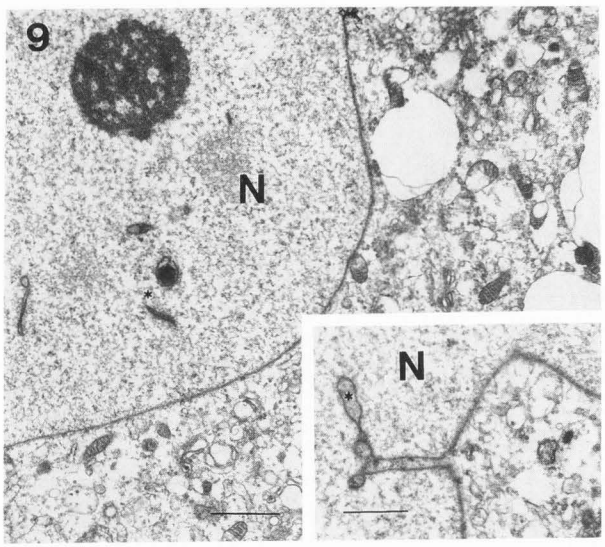
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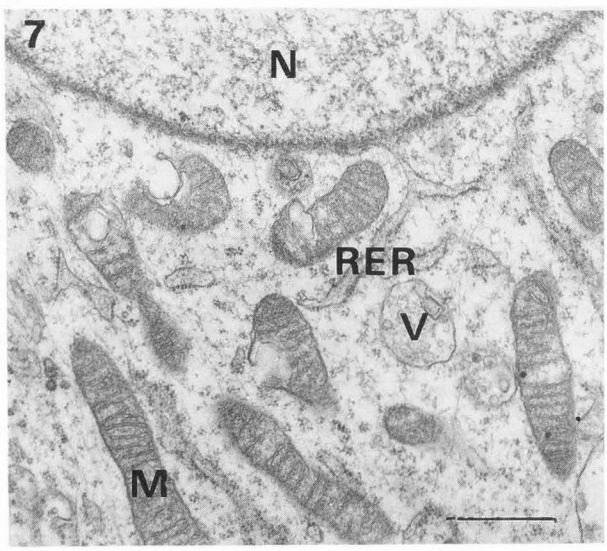
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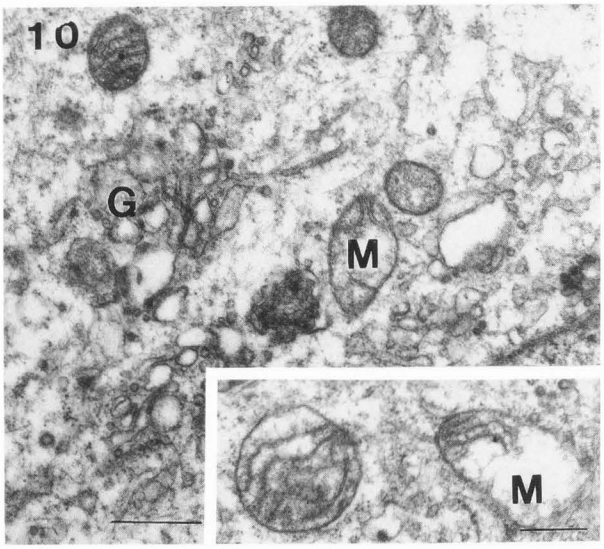
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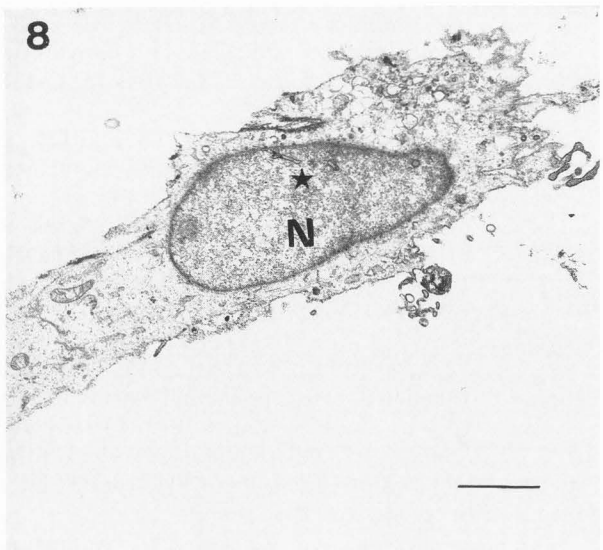
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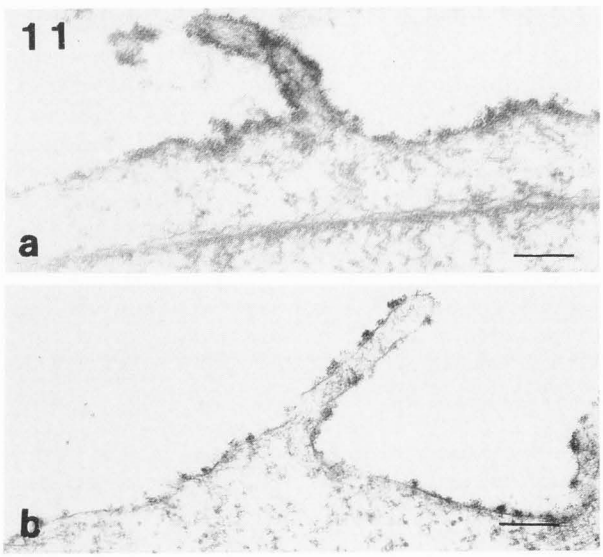
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Discussion with Reviewers

T.M. Seed: Besides the noted morphologic changes at

low HTO doses, are there any marked functional changes (i.e., change in cloning efficiency, cell-cycle alterations, etc)?
J.G. Szekely: Have you looked at growth kinetics or cell killing at these low HTO levels?
Authors: We have not investigated the change of cloning efficiency and growth kinetics after HTO treatment until now. We have not experienced cell killing at low HTO concentrations, either.

J.G. Szekely: I noticed that Figs. 3 and 4 show cells, which appear to be from a less dense culture and appear further from confluence than those in Figs. 1 and 2. Were the cells in the control and irradiated samples of the same cell density when the SEM preparations were made? If yes, were SEM pictures taken from parts of samples of the same cell density? If no, is there a chance that cell density influences the cytoplasmic extensions, cell-cell contacts and elevation of cell edges you report?
Authors: Cell number per area did not change after 37 kBq/ml or less concentrations of HTO as observed by light microscopy. The appearance of increased numbers of single cells can be explained by the shape changes (ruffling, rounding, blebbed surface) which are incident to decrease of cell surface area and loss of cell contacts.

K. Ijiri: It is surprising that morphological alterations of cells can be induced by quite a low dose of β-rays (2.3 mGy). The only thing I am worried about in the present experiment is the possible contribution of hydrogen peroxide (H_2O_2), which may have been accumulated in the HTO solution used. Did you check its concentration? Some Japanese scientists using *in vitro* system were once troubled with H_2O_2 contamination in HTO solution, and now they remove this substance by the catalase (ca. 10 units/ml or 1 μg/ml) treatment before use. Are there data that H_2O_2 also causes morphological changes observed here? I think it acts on the membrane system like other reactive oxygen species.
Authors: We did not check the H_2O_2 concentration in HTO solution, however, we have studied the effects of catalase as proposed by Dr. Ijiri. According to our recent experiments, the HTO solution (5 μCi/ml) previously treated with catalase (Sigma, 1 μg/ml) induced morphological changes identical to the effects caused by HTO without catalase treatment. Catalase treated solution without HTO did not cause any morphological effects. Further, it is known, that the exogenously added H_2O_2 can cause morphological changes similar to those we have observed (16, 18, 52, 53) in mM range and the radiation induced endogenous and exogenous free radicals play important role in radiation induced damage of membranes (43, 46, 60). However, the direct role of H_2O_2 generated from extracellular water by ionizing radiation is not clear, since its amount is very small (0.3 μM per Gy, according to Aebi HE, (1963. Detection and fixation of radiation-produced peroxide by enzymes. Rad. Res. Suppl. 3, 130-152.)

J.G. Szekely: 3T3 cells are frequently used in cellular transformation experiments as a model for carcinogenesis. Do you know if there is any transformation data on these cells at the doses you are using with HTO?
Authors: Little (text ref. 37) has published a paper on this topic. But the applied concentrations were higher (0.5 mCi/ml; i.e. 37 MBq/ml) than those we applied in our experiments.

J.G. Szekely: You suggest that deuterium effects on cell growth and morphology may be a model for what is happening with tritium. Are you aware of any reports in the literature which show non-radiation isotope effects of tritium in cellular systems?
Authors: There are a few reports on isotope effects of tritium in plant and animal cells and microorganisms as reviewed by Mathur-de Vré and Binet (41).

J.G. Szekely: Have you made any attempts to quantify the morphological effects you are reporting? Do the effects increase with dose? What is the lowest dose at which you have seen morphological changes?
Authors: Yes, we counted the number of fibroblastic forms of cells which have spread and the altered forms of them in control and HTO-treated cultures. The control cultures contained only 3-5% non-fibroblastic (elongated, rounded) forms of cells, the HTO-treated ones approximately 60-70%. This effect on shape did not change at higher (10 μCi/ml) dose. HTO in lowest (0.01 or 0.1 μCi/ml), concentrations did not cause any morphological changes.

T.M. Seed: Is the radiation dose delivered by HTO totally uniform in nature? Are there local intracellular 'hot spots'?
Authors: The HTO might show an inhomogeneous distribution in cells (40, 41), it is concentrated firstly in water pools of cells as well as in membrane bound water. Thus, there are possibilities of relatively higher concentrations (activity) of it on membranes.