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X-IRRADIATION-INDUCED CHANGES OF THE PRELYSOSOMAL AND LYSOSOMAL COMPARTMENTS AND PROTEOLYSIS IN HT-29 CELLS

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Abstract

As a consequence of external and internal ionizing radiation, lysosome-like bodies have been observed to increase both in size and number in some cell types. We investigated this process by morphological methods (electron microscopy, cationized ferritin uptake, acid phosphatase histochemistry, morphometry) in cultured HT-29 cells. In parallel with these studies, we measured the rate of protein degradation on the basis of ^{14}C-valine release from prelabeled cellular proteins. We found that at 2 and 4 Gy doses of X-irradiation the volume of the vacuolar (probably lysosomal) compartment increased without detectable changes of acid phosphatase activity. A 2 Gy irradiation dose did not change protein degradation rate. However, 4 Gy caused a significant inhibition of ^{14}C-valine release from prelabeled proteins. Our results indicate, that the radiation induced expansion of the lysosomal compartment is not necessarily accompanied by increased lytic activity of HT-29 cells.

Key Words: X-irradiation, protein degradation, lysosome, morphometry, acid phosphatase activity, endosome, autophagy, cationized ferritin uptake, cytoplasmic vacuolar compartments, HT-29 cells.

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Introduction

Lysosomes act as the primary component of the intracellular digestive system, and contain a large number and variety of digestive enzymes. Entry of proteins into lysosomes can occur via endocytotic and/or autophagic pathways. Elements of the endocytotic network appear as endocytotic and multivesicular bodies and contain material of plasma membrane and/or extracellular origin. According to different steps of the maturation process, they can be separated as early endosomes, late endosomes and prelysosomes/lysosomes. At the same time, they show compositional and biochemical differences with regard to pH gradients, proteolytic, esterolytic and fusogenic activities (Dunn, 1990a,b, 1994; Murphy, 1991; van Deurs *et al.*, 1993; Berg *et al.*, 1995; Ward *et al.*, 1995).

During autophagy various cellular constituents are ingested and digested by the cell's own lysosomal apparatus (De Duve and Wattiaux, 1966; Kovács and Réz, 1979; Pfeifer, 1987). Cellular autophagy can be carried out by different mechanisms, including the so called microautophagy and the classical or macro-autophagy (Dewaal *et al.*, 1986). During microautophagy the cellular constituents enter lysosomes by direct ingestion (De Duve and Wattiaux, 1966; Pfeifer, 1987), and they form multivesicular bodies. Segregation of cytoplasmic regions via special isolating cisternae is the first step of macroautophagy. The further maturation of these newly formed autophagosomes into autolysosomes is the consequence of their fusion with Golgi-originated primary lysosomes and/or other secondary lysosomes (De Duve and Wattiaux, 1966; Dunn, 1990a,b, 1994; Kovács and Réz, 1979; Pfeifer, 1987; Sakai *et al.*, 1989).

The various elements of the endocytotic and autophagic vacuolar compartments differ in their fusogenic activity. These two pathways meet at a common endpoint, and the resulting compartment can contain both originally cytoplasmic and extracellular material (Sakai *et al.*, 1989; Dunn, 1994; Knight *et al.*, 1995).

As a consequence of external and internal ionizing irradiation lysosome-like bodies have been observed to

increase both in size and number in some cell types (Table 1). The increase of the number of lysosomes was shown to be accompanied by an increase of their acid phosphatase (AcPhase) activity (Hamberg, 1983; Conti *et al.*, 1974; Reynolds and Wills, 1974).

Several reports show that lysosomal enzymes may appear in the cytosol or in extracellular fluid during the postirradiation period (Hugon and Borgers, 1966; Brandes *et al.*, 1967; Aikman and Wills, 1974; Snyder, 1977; Snyder and Eklund, 1978; Clark and Willis, 1980; René *et al.*, 1971; Harris, 1970).

In spite of the fact that the radiation-induced increase in the fractional volume of the lysosomal compartment is interpreted by most authors as a sign of increased lysosomal activity, or in certain cases, of enhanced autophagocytosis, other explanations for this phenomenon also seem to be possible. It is a well documented fact that the slowing down of the turnover of autophagic vacuoles/lysosomes may also lead to the increase in the volume of this compartment due to overload by undigested macromolecules (Hirsimaki *et al.*, 1976; Amenta *et al.*, 1977; Marzella and Glaumann, 1980; Kovács, 1983; Kovács *et al.*, 1985, 1986; Réz *et al.*, 1990; Fosse *et al.*, 1995).

In order to clarify these questions, we investigated in the present study the X-ray induced volume changes of endocytotic and autophagic vacuolar compartments and secondary lysosomes by morphometrical methods, and in parallel we measured the rate of protein degradation on the basis of ^{14}C valine release from prelabeled proteins of HT-29 cells.

The connection between the vacuolar compartments and the extracellular space was studied by use of cationized ferritin (CF) labeling. The lysosomal nature of any vacuole was revealed by cytochemical demonstration of the lysosome-specific marker enzyme acid phosphatase.

Materials and Methods

Cells

The human colon carcinoma-derived cell line HT-29 originally established by Fogh and Trempe (1975) was obtained from Public Health Laboratory Service (Salisbury, U.K.).

Confluent cultures of the HT-29 cells were incubated in cell culture flasks in 8 ml RPMI medium (Gibco, Paisley, Scotland, U.K.) complemented with 10% foetal calf serum (FCS, Gibco) and antibiotics at 37°C in 5% CO_2.

X-irradiation

X-irradiation was performed with 2.0 and 4.0 Gy doses by a THX-250 X-ray source (Medicor, Budapest, Hungary). Conditions were: 200 kV, half value layer: 1.0 mm copper, source-surface distance: 90 cm, dose rate: 0.317 Gy/min. Irradiation was carried out at room temperature in culture medium. Cell cultures were fixed for electron microscopy and acid phosphatase cytochemistry. Protein degradation was measured immediately, and 2, 4, 6 and 24 hours after irradiation. Cationized ferritin was applied to label vacuoles of endocytotic origin to unirradiated samples and to cells 4 hours after 4 Gy dose irradiation.

Electron microscopy

The cells were scraped from culture flasks and were fixed as a suspension for 1 hour at 4°C in 0.1 M phosphate buffered 2.5% glutaraldehyde (pH 7.3), postfixed in 1% OsO_4, dehydrated with ethanol and embedded in Durcupan (Fluka, Switzerland). The samples were cut with diamond knives on an LKB (Bromma, Sweden) Ultratome, and the sections were stained with lead citrate and uranyl acetate and examined in a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan).

Histochemistry

Acid phosphatase (AcPhase) histochemistry After glutaraldehyde fixation, the cell suspension was washed in 0.1 M Na-cacodylate buffer (pH 7.4), followed by incubation for 1 hour, at 37°C in a mixture of 0.1 M acetate buffer (pH 5.0) containing 1 mM β-glycerophosphate, 2 mM $CeCl_3$ and 5% saccharose (Zhang *et al.*, 1991). Then the cells were embedded in 1% agar, postfixed with 0.5% OsO_4 and 4% uranyl-acetate, dehydrated and embedded in Araldite. The sections were counterstained with uranyl-acetate.

Cationized ferritin (CF) labeling Cells were incubated in the presence of 0.25 mg/ml CF (Sigma, St. Louis, MO) added to culture medium for 5, 30, 60 and 120 minutes. (A brief preliminary survey showed that 120 minutes samples showed the most reliable results). This was followed by processing for electron microscopy as described above. The ultrathin sections were not counterstained by lead citrate or uranyl acetate.

Morphometric analysis For quantitative electron microscopic examination, ultrathin sections were cut at a thickness of about 50 nm. All samples were contrasted by uranyl acetate and lead citrate. Three samples were processed from each experimental group. Electron micrographs were taken randomly from each sample at a primary magnification of 10,000. A 1000 μm^2 cytoplasmic area was analyzed from each experimental group by a computer-assisted morphometrical method. Electron micrographs were coded and were evaluated blindly using a personal computer assisted digitalizer tablet and a self-developed software. Nucleus free cytoplasmic area and investigated intracellular compartments were circumscribed using a digitalizer pen, and each compart-

Table 1. Irradiation induced alteration of autophagic vacuole/lysosomal compartment in various cells. BHK cell = baby hamster kidney cell; KB cell = human oral cavity epidermoid carcinoma cell.

Cell type	Irradiation	Compartments (increased in volume)	Reference
rat lung	X-ray	lysosomes	Salovsky and Shopova, 1992
granular pneumocytes	X-ray, 15, 20 Gy, 3-6 hours	small autophagic vacuoles	Maisin, 1970
membranous pneumocytes		autophagic vacuoles, multivesicular bodies	
endothelial cells		large autophagic vacuoles	
rat alveolar macrophages	thermalised neutron	lysosomes*	Morris *et al.*, 1989
epithelium of duodenal crypt	X-ray	lysosomes	Hugon and Borgers, 1966
mouse hepatocytes	X-ray, mixed γ-neutron	lysosomes myelin figures	René *et al.*, 1971 René and Evans, 1970
thyroid	I^{131}	autophagic vacuoles	Sobel, 1964
cultured neuroblastoma cells	X-ray	autophagic vacuoles and multivesicular bodies, heterophagy	Hamberg *et al.*, 1977, Hamberg, 1983
mouse gland carcinomas	X-ray	lysosomes	Brandes *et al.*, 1967
Oral epithelium (rat)	X-ray, 50 Gy, 26-50 hours	autophagic vacuoles	Liu *et al.*, 1977
jejunum, Rhesus monkey	32 MeV protons	giant lysosomes	Ghidoni and Campbell, 1969
rat dorsal ganglia neurons	X-ray	autophagic vacuoles	Masurovsky *et al.*, 1967
pituitary adenomas	radiotherapy	autophagic vacuoles	Betzold *et al.*, 1992
hippocampus	^{60}Co, 1 Gy, prenatal irradiation	autophagic vacuoles	Hamdorf *et al.*, 1990
cultured BHK cells	X-ray	autophagic vacuoles	Conti *et al.*, 1974
Chang liver cells	X- or β-irradiation	autophagic vacuoles, lysosomes	Montgomery *et al.*, 1964
cultured KB cells	X-ray	autophagic vacuoles	Lane and Novikoff, 1965
HeLa cells	X-ray	lysosomes	Reynolds and Wills, 1974
glial cells	γ-rays	autophagic vacuoles	Ostenda, 1973
splenic macrophage from rat	γ-rays	hetero- and autophage vacuoles	Piao *et al.*, 1983
HT-29 cell	X-ray	lysosome compartment*	Somosy *et al.*, present paper
macrophages	incorporated ^{241}Pu particles	autophagic vacuoles	Rahman and Lindenbaum, 1964
rat macrophages	incorporated ^{241}Pu particles	autophagic vacuoles	Miller and Bowman, 1983
cochlea of the guinea pig	fast neutron, 2, 6, 10, 15 Gy	autophagic vacuoles	Kim and Shin, 1994

*morphometrical evaluations

ment was marked by a corresponding color-code. All evaluated images were downloaded together with a configuration file, which contained the magnification value and the different color codes. Bitmap images were evaluated using a self-developed software. The volume fraction (V_v) of different kinds of cytoplasmic vacuoles was calculated by relating the area of vacuole profiles to the total area of cytoplasm, and all results were evaluated by a commercially available statistical software. Average values and corresponding confidence intervals were calculated. Statistical analysis was carried out by the χ^2 test. The cellular distribution of the studied vacuoles was found to be different from normal distribution. Therefore, the percentile distribution of relative volume of each vacuole type in each group of cultures was calculated and compared to the percentile distribution of the corresponding vacuole in the unirradiated controls.

Endogenous protein degradation For the measurement of protein degradation U-^{14}C-valine (10 μCi in 200 μl) was added to each flask of cultures. After the 24 hour long labelling incubation (37°C, 5% CO_2), the medium was removed, and flasks were washed free of soluble radioactivity 3 times with RPMI 1640 solution. 5 ml new medium containing 5 mmol/l cold valine (Sigma) was added to the cells for chase, and a second incubation was carried out for 2, 4, 6 and 24 hours to allow for the accumulation of proteolytically released free valine. Three parallel series of measurements with 3 parallel samples in each case were processed. Before the second incubation, cell cultures were X-irradiated with 2 or 4 Gy (X-ray source: THX type 250; Medicor). Controls were not irradiated and samples of "0 minutes" were taken before the second incubation. At the end of the second incubation, cells were scraped off the bottom of the flasks and removed together with the incubation fluid. Cell suspensions were mixed with 10% perchloric acid (PCA; final concentration 2%). The mixture was kept on ice for 10 minutes and centrifuged with 3000 rpm for 15 minutes at 4°C afterwards.

Radioactivity of aliquots of supernatants were measured in a liquid scintillation counter (LKB Rackbeta) with Sigma-Fluor liquid scintillation cocktail (Sigma).

The precipitate was washed three times with cold 2% PCA and dissolved in 0.1 mol/l NaOH containing 0.4% deoxycholic acid. The solution was acidified (to pH 5.0), and aliquots were used for radioactivity measurement. Ultraviolet (UV) absorption (260 and 280 nm) of dissolved precipitate was also measured. Protein content was determined according to the Bradford Coomassie blue reaction (Bradford, 1976).

Cell viability Cellular viability was determined by the Trypane-blue exclusion test. 500 cells were counted for each single time point in control samples and after irradiation. Decrease in cellular viability could not be observed in control cultures and up to 24 hours after 2.0 Gy doses irradiation. However, 4% of cells died after 24 hours following a 4.0 Gy dose.

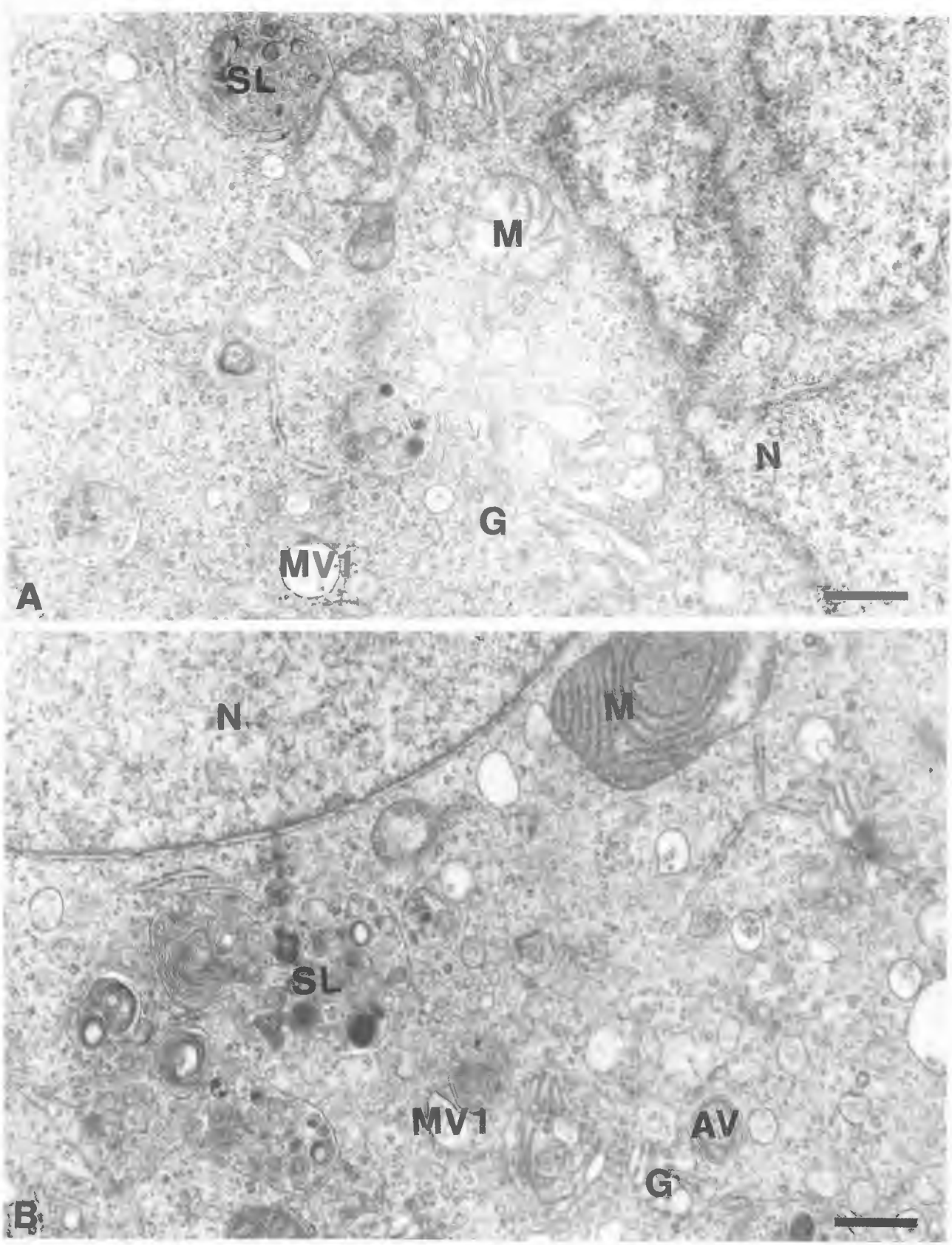
Figure 1. (*on facing page*) Electron micrographs of unirradiated control (**A**) and X-ray-irradiated (2 Gy, 4 hour) HT-29 cells (**B**). The cells have well developed Golgi-apparatus (G) and different kinds (see text and Fig. 2) of vacuoles. Note the complex vacuoles (SL) in X-irradiated cells, which are as a rule larger than those in irradiated cells. AV = early autophage vacuole, M = mitochondria, N = nucleus, MV1 = multivesicular body 1, SL = complex multivesicular body. Bars = 0.5 μm.

Results

The undifferentiated HT-29 cells in confluent culture had mitochondria of variable size, a few cisternae of granular endoplasmic reticulum, a well developed Golgi complex, and various kind of vacuoles, vesicles, including secretion vesicles, multivesicular bodies and different elements of the lysosomal system (Fig. 1A). The irradiated cells contained elevated number and/or larger autolysosome like bodies (Fig. 1B). The autophagic/lysosomal vacuoles showed non-random distribution both in control and irradiated HT-29 cells, and they were preferentially located around the nucleus and in the vicinity of Golgi stacks (Fig. 1B).

In this study, five types of vacuoles of the autophagic/lysosomal compartment were identified on the basis of their morphology, AcPhase activity and CF labeling (Fig. 2). The autophagic vacuoles (AV) (Fig. 2A) were defined as bodies bordered by smooth single or double isolating membranes and containing undigested cytoplasmic material. AcPhase activity and accumulation of CF could not be observed in this type of vacuoles.

Two types of secondary lysosomes (SL1 and SL2) were distinguished in this study. SL1 appeared as complex multivesicular bodies both in control and irradiated cells (Figs. 1 and 2B-D). They contained numerous vesicles of various diameter and membrane whorls. They could be labeled by CF (Fig. 2C) and were AcPhase positive (Fig. 2D). Our electron micrographs suggest that they can easily fuse with each other to form complex vacuoles (Fig. 2M). The other category of secondary lysosomes (SL2) (Figs. 2E and G) contained electron dense matrix which may be of extracellular origin, as revealed by cationic ferritin labeling (Fig. 2F), and they are AcPhase positive as well (Fig. 2G).


SL
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MV1
A
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B

In HT-29 cells, two types of multivesicular (MV) bodies could be distinguished. The MV1 bodies were electron lucent vacuoles containing vesicles more or less uniform in diameter, often located beneath the membrane bordering the vacuole (Fig. 2H). This type of vacuole could not be labeled by CF (Fig. 2I) and showed AcPhase activity (Fig. 2J). The MV2's were densely packed with small vesicles of uniform diameter which can bind CF (Fig. 2L) and showed AcPhase activity (Fig. 2J).

According to our morphometric evaluation, the volume fraction of AV, SL2 and MV1 vacuoles remained unchanged upon irradiation (Table 2). However, this treatment caused an increase of volume fraction and size of SL1 vacuoles (Fig. 1B, Table 2), with concomitant decrease of the volume fraction of MV2 (Table 2). As shown in Table 2, these changes are significant at the 4 Gy dose. A moderate, but significant, increase of the volume fraction of SL1 was observed after 4 hours at 2 Gy radiation dose; however, the volume fraction of other vacuoles remained unchanged under these conditions.

The cisternae and tubules of trans-Golgi network and the SL and MV types of vacuoles expressed easily detectable AcPhase activity in the controls and after 2 Gy irradiation (Figs. 2D,G and 3A). However, after 4 Gy radiation dose AcPhase activity was rarely detected in the expanded compartment of complex multivesicular bodies (SL1) (Fig. 3B).

Figure 4 shows the effect of irradiation on endogenous protein degradation in HT-29 cells. Accumulation of acid soluble radioactivity in the culture medium indicates that proteolysis proceeds both in the control and 2 Gy irradiated cells at similar rate. However, a strong and significant inhibition of release of ^{14}C valine from radioactive cellular proteins was observed upon 4 Gy irradiation at 2-24 hours (Fig. 4).

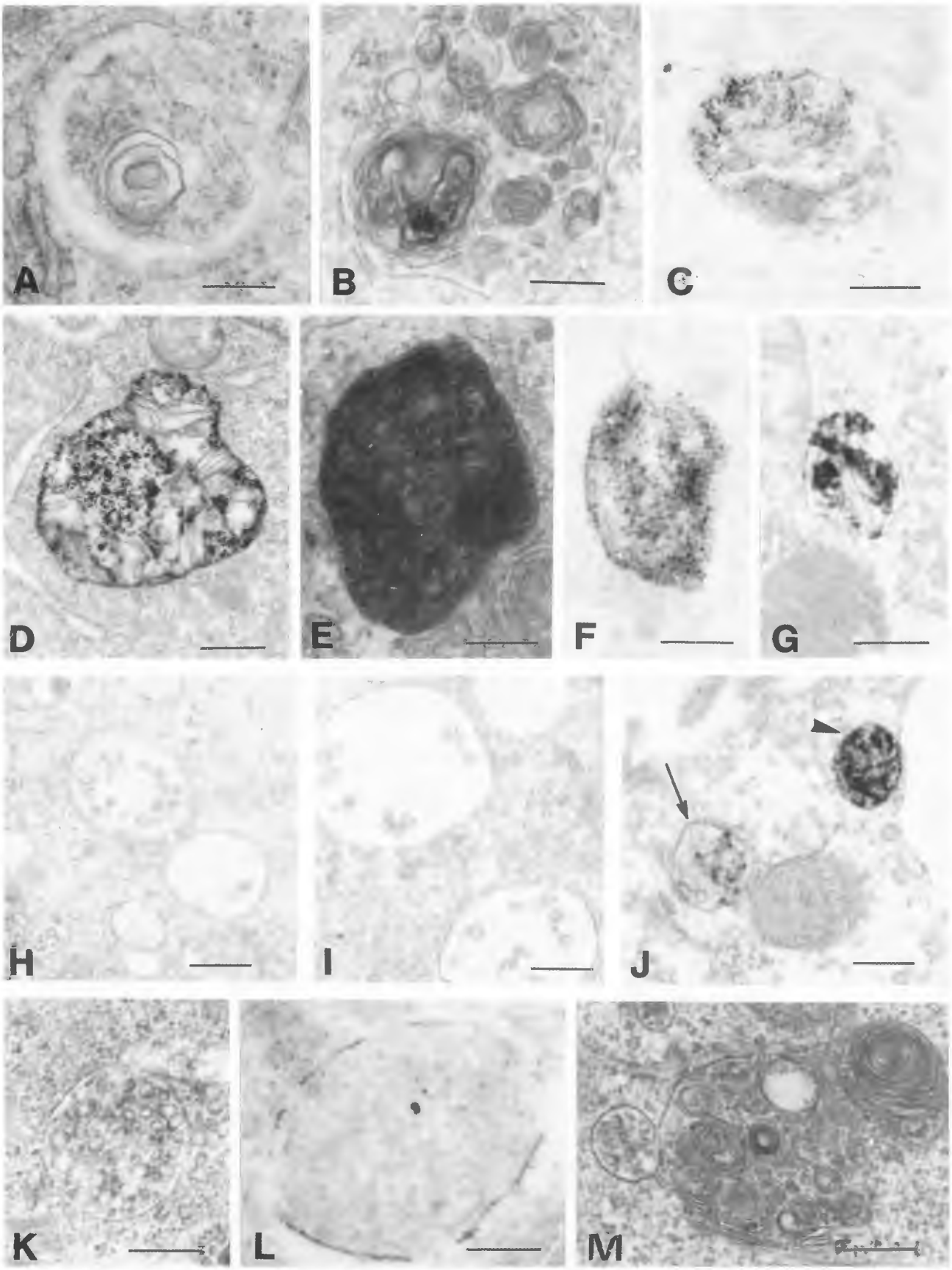
Discussion

Five different types of endosome/lysosome like vacuoles of HT-29 cells were examined in this study. Four of them (SL1 and 2, MV1 and 2) showed AcPhase activity, and three of them (SL1 and 2 and MV2) were labeled by extracellularly added CF. These data show, in agreement with earlier data (Thyberg *et al.*, 1980; Hamberg, 1983; Mosselmans *et al.*, 1984; Coleman and Hand, 1987; Atwa *et al.*, 1990), that they belong to the endosome/lysosome system of HT-29 cells and that they are accessible to externally applied markers. However, the mechanisms by which these compartments communicate with each other and the mode of the transfer of extracellular cargo from one compartment into the other remain to be elucidated, and these questions were out of the scope of this study.

Figure 2. (*on facing page*) Morphology and histochemistry of cytoplasmic vacuoles investigated in this study. (**A**) Early autophagic vacuole. (**B**) Complex secondary lysosome containing vesicles of various diameter and membrane whorls. This type of vacuoles accumulates CF (**C**) and shows AcPhase activity (**D**). (**E**) Compact electron dense residual body, which can be labeled by cationized ferritin (**F**) and are AcPhase positive (**G**). (**H**) Multivesicular body containing loosely arranged clear vesicles, which are not labeled by cationized ferritin (**I**), however, they show AcPhase activity (**J**, arrow). (**K**) Multivesicular body characterized by densely packed vesicles, which show AcPhase activity (J, arrowhead) and can be labeled by CF (**L**). (**M**) A possible fusion of complex secondary lysosomes and multivesicular body vacuoles. Bars = 0.2 μm.

The early forms of autophagic vacuoles were devoid of AcPhase activity and inaccessible to CF. These observations are in good agreement with data of other reports (De Duve and Wattiaux, 1966; Kovács and Réz, 1979; Hamberg, 1983; Pfeifer, 1987; Sakai *et al.*, 1989; Dunn, 1990a,b, 1994), showing that the nascent autophagosomes acquire acid phosphatase activity in a later step by fusion with pre-existing members of endosome/lysosome system of the cell.

The main finding of this study is that the volume fraction of the SL1 secondary lysosomes of HT-29 cells greatly expands upon nonlethal doses of irradiation. It is well known from literature that members of the endosome/lysosome system of various cells sensitively respond by volume increase or decrease to different physiological stimuli (Ericsson, 1969; Szego and Seeler, 1973; Pfeifer, 1987; Kirk and Murphy, 1991), to certain pathological conditions (Ericsson, 1969; Aula *et al.*, 1975), and to a wide variety of chemical and physical agents (Ericsson, 1969; Amenta *et al.*, 1977; Kovács and Réz, 1979; Marzella and Glaumann, 1980; Kovács, 1983; Kovács *et al.*, 1985, 1986; Réz *et al.*, 1990). As shown in Table 1, many authors found an increase in the volume of lysosome-like compartments in various cells following irradiation, and this expansion was often interpreted as sign of increased lysosomal activity. However, the actual size of the endosome/lysosome compartment is determined by the rate of influx (e.g., by the rate of autophagic sequestration and of endocytotic uptake of extracellular components) and by the rate of the efflux (i.e., by the rate of degradation of sequestered and/or endocytosed macromolecules) of degraded material. Therefore, its expansion may be explained either by increased uptake of intra- and /or extracellular macromolecules, or by an overload due to diminished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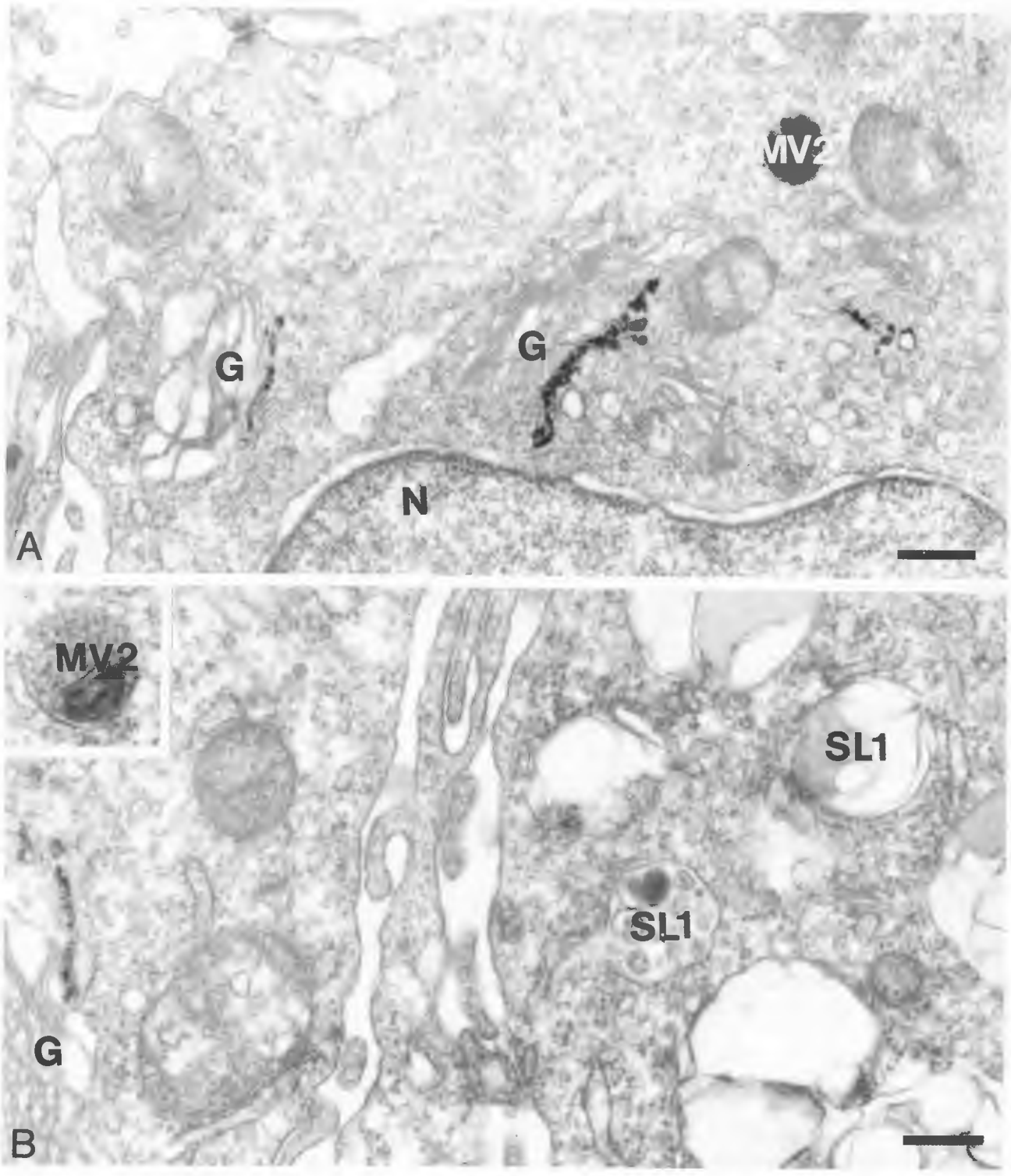

Figure 3. Acid phosphatase activity is detected in trans-Golgi cisternae (G) and in secondary lysosomes (MV2) of unirradiated control cells (A). (B) After irradiation (4 Gy, 24 hours), the enzyme activity disappears from the complex secondary lysosomes (SL1), however, it can be detected in the trans-Golgi cisternae (G) and MV2 type bodies. Bars = 0.5 μm (A) and 0.2 μm (B).

Table 2. The fractional cytoplasmic volume (V_v) of autophagic vacuoles (AV), secondary lysosomes (SL1 and 2) and multivesicular bodies (MV1 and 2) of unirradiated and irradiated HT-29 cells.

X-Ray Dose [Gy]	hrs. after irradiation	AV1	Confidence	SL1	Confidence	SL2	Confidence
0	0	0.29%	(0.15% - 0.42%)	2.37%	(1.49% - 3.25%)	1.61%	(0.91% - 2.31%)
2	2	0.31%	(0.15% - 0.47%)	3.00%	(0.57% - 5.43%)	0.63%	(0.05% - 1.21%)
2	4	0.41%	(0.20% - 0.62%)	5.47%	(2.67% - 8.27%) *	1.96%	(0.80% - 3.11%)
2	24	0.31%	(0.00% - 0.63%)	2.23%	(0.33% - 4.14%)	2.86%	(0.45% - 5.27%)
4	2	0.48%	(0.22% - 0.73%)	5.21%	(3.16% - 7.26%)	2.60%	(0.68% - 4.52%)
4	4	0.54%	(0.22% - 0.85%)	6.77%	(4.64% - 8.89%) **	1.28%	(0.75% - 1.82%)
4	24	0.16%	(0.03% - 0.30%)	6.43%	(4.40% - 8.45%) **	1.72%	(0.90% - 2.53%)
		MV1	Confidence	MV2	Confidence		
0	0	0.43%	(0.07% - 0.78%)	2.20%	(1.59% - 2.81%)		
2	2	0.16%	(0.00% - 0.50%)	1.95%	(1.21% - 2.68%)		
2	4	0.25%	(0.00% - 0.52%)	1.46%	(0.88% - 2.05%)		
2	24	0.20%	(0.00% - 0.89%)	1.57%	(0.01% - 3.12%)		
4	2	0.34%	(0.00% - 0.80%)	2.64%	(1.85% - 3.42%)		
4	4	0.36%	(0.02% - 0.71%)	1.56%	(1.04% - 2.09%)		
4	24	0.33%	(0.18% - 0.47%)	1.17%	(0.74% - 1.60%) *		

** =$p<0.05$

* =$p<0.05$ if only size distribution is linearized to size distribution of unirradiated controls

activity of lysosomal enzymes (Hirsimaki *et al.*, 1976; Amenta *et al.*, 1977; Marzella and Glaumann, 1980; Kovács *et al.*, 1985, 1986; Ballard, 1987; Réz *et al.*, 1990; Fosse *et al.*, 1995). Our data on significant inhibition of release of ^{14}C-valine from prelabeled cell proteins following 4 Gy irradiation and on the disappearance of histochemically detectable AcPhase activity from the complex SL1 type vacuoles indicate that the proteolytic activity of lysosomes decreased in the irradiated cells and strongly suggest that accumulation of undegraded and/or partially digested substances lead to the increase of volume fraction of complex secondary lysosomes. After 2 Gy X-irradiation, significant volume increase of the SL1 compartment was detected without changes in protein degradation (^{14}C-valine release) and observable change in AcPhase activity. This lack of increase of protein degradation, in spite of the expansion of SL1, may also be interpreted as being the result of accumulation of undegraded substances in this compartment. However, it must be kept in mind that the expansion of the lytic compartment following irradiation is a complex dose dependent process and factors other then accumulation of undegraded substrates in the lysosomes (e.g., enhanced endocytotic activity) should also be taken into consideration.

The lysosomal uptake of macromolecules depends on the integrity of cytoskeletal system (Sakai *et al.*, 1989), and it is known that both autophagic and heterophagic processes are influenced by cyclic nucleotides and cytoplasmic calcium ion concentration (Pfeifer and Guder, 1975; Gordon *et al.*, 1993). In an earlier study, we showed that the actin network disassembles in HT-29 cells upon irradiation (Somosy *et al.*, 1995), and it is also well known that the ionizing radiation disturbs the Ca^{2+} homeostasis in these cells (Todd and Mikkelsen, 1994) and modified the intracellular level of cyclic nucleotides (Somosy *et al.*, 1988; Sorokina *et al*, 1992). It seems plausible to propose that alteration of these regulatory mechanisms also contributed to changes of lysosomal system observed in this study.

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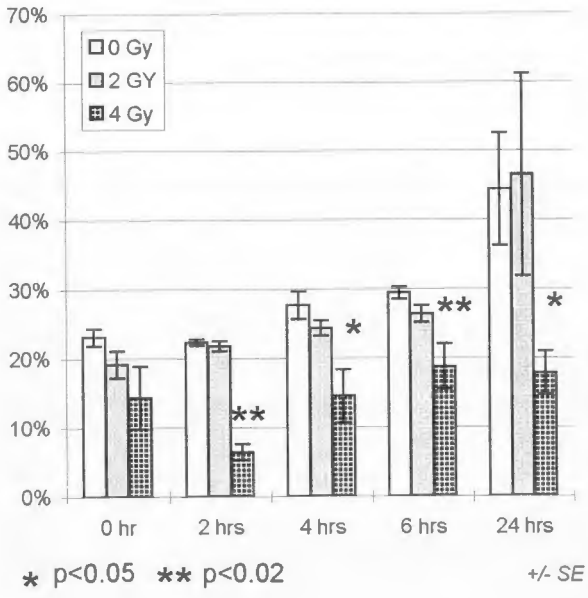

Figure 4. The time course (hours) and dose dependence of degradation of endogenous proteins following X-irradiation. Degradation was determined by measuring the PCA soluble radioactivity released from cells prelabeled with ^{14}C-valine into the medium. Data are expressed as a percentage of acid precipitable activity (± SE) at 0 minutes. As shown on this Figure, the valine release significantly decreased upon 4 Gy irradiation.

Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Hungarian Army Institute for Public Health and Military Medical Research, respectively).

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Discussion with Reviewers

M.E.C. Robins: It is not clear what the HT-29 cells are. Which organ/tissue do they come from? Why were they selected?

L.S. Yasui: Why were confluent cultures of HT-29 cells used in this study?

Authors: The human colon carcinoma-derived cell line HT-29 was originally established by Fogh and Trempe (1975). This cell type is a valuable model for study of intestinal epithelial cell differentiation and cellular autophagy. The culture in confluent state consists of cells more uniform in respect to morphology and radiosensitivity than the counterpart in the state of logarithmic growth.

L.S. Yasui: Irradiation of the HT-29 cells with 2 Gy and 4 Gy should produce significant cell killing in exponentially growing cells as measured by clonogenicity. The clonogenicity in plateau phase cells may be greater than the exponential cells, but it should be determined if the authors are going to make conclusions based on cell survival. The trypan blue dye exclusion only detects cells that have disrupted plasma membranes or lysed cells.

Authors: That is true. However, for our experiments and data on cells remaining alive during the first 24

hours after irradiation were relevant.

M.E.C. Robins: Was it not possible to quantify the radiation-induced decrease in AcPhase activity observed after 4 Gy?
U. Brunk: The cytochemical demonstration of acid phosphatase shows diminished enzyme activity in secondary lysosomes after 4 Gy of irradiation, although preserved activity in the Golgi zone. Is the enzyme not transported from the Golgi, or is it inactivated? Biochemical measurements should have been done as well.
Authors: Yes, we plan to address this question in the future.

J. Trosko: The data presented do not fully support the hypothesis (i.e., radiation increases prelysosomal proteolytic activity).
Authors: We did not measured the proteolytic activities of prelysosomal and lysosomal compartments separately. What we actually found was the dose dependent decrease of overall proteolytic activity of the cells following 4 Gy irradiation dose measured as PCA soluble radioactivity released into the medium from cells prelabeled with ^{14}C-valine and a concomitant increase of the fractional volume of lysosome compartment measured by morphometry, and a decrease of histochemically detectable acid phosphatase activity in prelysosomal and lysosomal compartments. We think that these data strongly suggest that the expansion of lysosomal compartment following irradiation is a least partly due to accumulation of undegraded material in the lysosomes.

L.S. Yasui: Why were the CF experiments done?
Authors: Cationized ferritin was used in our experiments as a tool to identify the types of vacuoles observed in the cells. It is well known that this substance can label vacuoles of endocytotic origin and secondary lysosomes, however, it does not enter early autophagic vacuoles.

U. Brunk: How do the authors explain the observation that the measurements of protein turnover show increases in control cells after 24 hours?
Authors: Data on the release of ^{14}C-valine from prelabeled protein show the accumulation of acid soluble radioactive amino acid in the medium. Ongoing protein degradation, therefore, inevitably leads to increased values with increasing time. From the point of view of our conclusions, the important fact is that in spite of the expansion (indicated by morphometry) of certain degradative elements of the vacuolar (lysosomal) compartment in the irradiated samples (especially in the 4 Gy treatment), no significant increase (2 Gy), but rather a decrease (4 Gy) was seen in the amount of accumulated radioactive valine.