Localization of Skeletal-Imaging ^{99m}Tc Chelates in Dead Cells in Tissue Culture: Concise Communication

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Screening new agents for imaging myocardial infarcts and investigating the mechanism of cellular uptake and subcellular localization of these agents prompted us to develop a necrosis model in tissue culture. Live cells obtained from exponentially growing cultures and dead cells obtained from nutritionally depleted plateau-phase cultures were incubated with ^{99m}Tc-pyrophosphate, ^{99m}Tc-hydroxyethylene diphosphonate (^{99m}Tc-HEDP), ^{67}Ga-citrate, and ^{45}Ca-HEDP. For the bone-seeking ^{99m}Tc chelates, the ratio of dead to live cell radioactivity was 15–20 after 1 hr of incubation. On the other hand, no preferential binding of ^{67}Ga-citrate was observed, indicating that the mechanism of ^{67}Ga localization in infarcts is different from that of the ^{99m}Tc chelates. The dead cells accumulated only twice as much ^{45}Ca-HEDP or ^{45}Ca-pyrophosphate as did live cells, possibly due to the carrier effect of calcium in the medium. The receptors for ^{99m}Tc chelates could be partially blocked by preincubating the dead cells with microgram amounts of Na-HEDP. Heat-denaturation of live cells also increased the localization of ^{99m}Tc-HEDP in the cells, and this uptake was proportional to the duration of heating. Bone-seeking ^{99m}Tc chelates could be used for marking dead cells instead of conventional staining methods.

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The development of ^{99m}Tc-tetracycline (*1,2*) and other ^{99m}Tc chelates (*3,4*) and their successful use in delineating myocardial infarcts, led us to develop an in vitro cellular necrosis model in tissue culture (*5–7*). In this investigation, we have used this model to screen radiopharmaceuticals, specifically ^{99m}Tc chelates, for their ability to label necrotic cells. The radiopharmaceuticals evaluated were double-labeled with beta- and gamma-emitting tracers and their subcellular distributions were determined by differential centrifugation. The effect of receptor-site blockade was studied by preincubation with unlabeled chelating agents. We also chose ^{45}Ca to mark dead cells since Shen and Jennings (*8*) showed that ^{45}Ca accumulates in the mitochondria of dead cells in infarcted myocardium. To study the relation of irreversible binding with the denaturation of macromolecules, we used thermal denaturation of cells, followed by incubation with bone-seeking tracers. We found this simple model very useful in screening diagnostic agents for their ability to localize in myocardial infarcts and in studying their site of intracellular localization.

MATERIALS AND METHODS

Chang liver cells (Lich) were grown in monolayer culture at 37°C in an atmosphere of 5% CO_2 in plastic flasks using a modified Eagle's basal medium supplemented with 10% heat-inactivated calf serum. The methods of obtaining dead cells from plateau-phase cultures and live cells from exponen-

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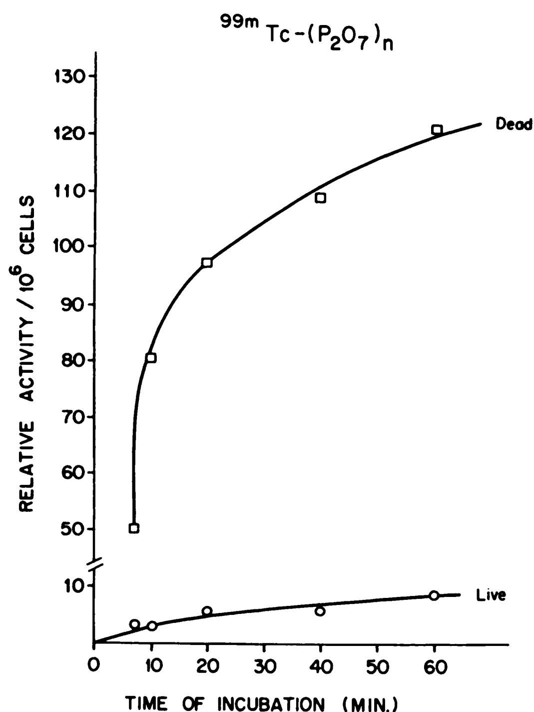

FIG. 1. Labeling of live and dead cells with ^{99m}Tc-pyrophosphate.

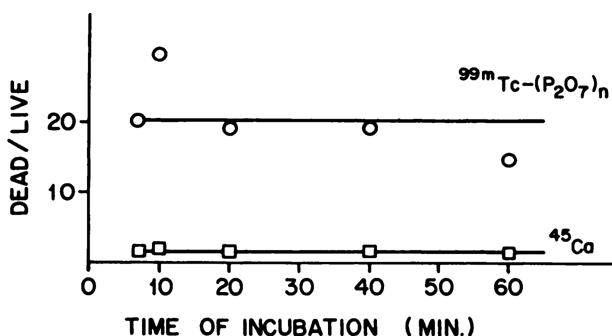

FIG. 2. Variation of live-to-dead-cell activity ratio with time of incubation for ^{99m}Tc-pyrophosphate and ^{45}Ca-pyrophosphate.

tially growing cultures have been described previously (*5*). Stannous pyrophosphate (Mallinckrodt/Nuclear, St. Louis, Mo.), Sn-HEDP (Procter & Gamble, Cincinnati, Ohio), ^{67}Ga-citrate (New England Nuclear Corp., North Billerica, Mass.), Na-HEDP (Monsanto, Miamisburg, Ohio), ^{45}Ca (New England Nuclear), and Eagle's minimum essential medium (Grand Island Biological Co., Berkeley, Calif.) were used in this investigation. The live and dead cells were resuspended for 5–60 min in 5 ml of medium containing 15 μCi of ^{99m}Tc-pyrophosphate, ^{99m}Tc-HEDP, or ^{67}Ga-citrate. In experiments where double-labeling with ^{99m}Tc and ^{45}Ca was performed, the ^{99m}Tc chelate was mixed with ^{45}Ca prior to incubation with the cells. After the incubation, the cells were washed three times with isotonic saline solution to remove unbound label. The activities in aliquots of medium, washings, and cells were then assayed with a gamma well counter. For the determination of ^{45}Ca by beta-counting, the cells and washing fractions were treated with 1 ml of Protosol (New England Nuclear) and transferred to liquid-scintillation vials by repeating washing and dispersing with a vortex mixer. Ten millimeters of liquid-scintillation cocktail (Aquasol, New England Nuclear) was added, and the samples were dark-adapted in the refrigerator overnight. After counting with a liquid scintillation counter (Packard, Downers Grove, Ill.), the β and γ activities per million live and dead cells were determined.

In the subcellular distribution studies (*9*), labeled and washed cells were homogenized in 3 ml of 0.25 *M* sucrose solution. The homogenates of live and dead cells in duplicate were centrifuged at 750 g for 10 min. The sediments were resuspended and centrifuged for 10 min at the same speed to obtain the nuclear fraction. The supernatants were mixed and centrifuged at 7,200 g for 10 min. The procedure was repeated at 24,000 g for 15 min and the two sediments were combined to get the mitochondrial activity. Finally, for the soluble fraction, the supernatant was spun at 54,000 g for 40 min. The activity in this sediment corresponded to microsomes. The β and γ activities in these fractions were determined as described above.

In the thermal denaturation procedure, the live cells were heated at 50°C for 10 or 30 min and then

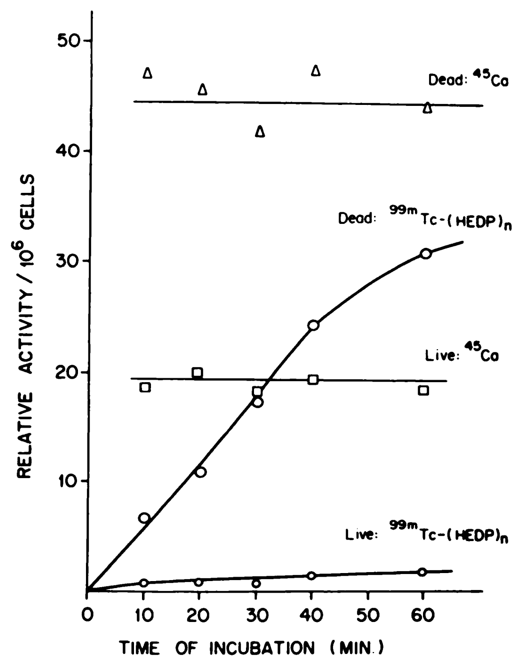

FIG. 3. Labeling of live and dead cells with ^{99m}Tc-diphosphonate and ^{45}Ca-diphosphonate.

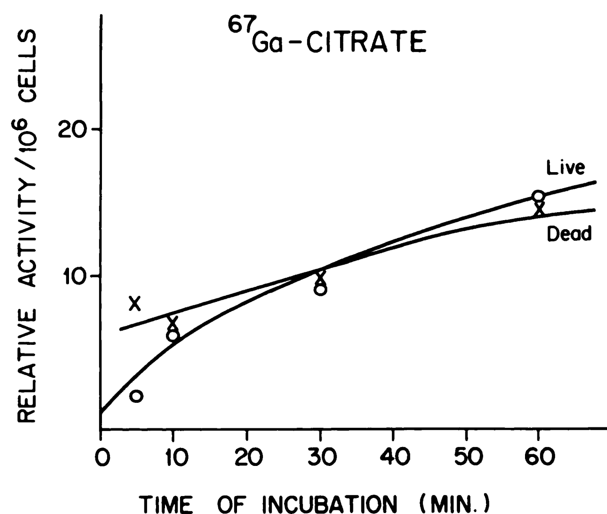

FIG. 4. Labeling of live and dead cells with ^{67}Ga-citrate.

incubated with ^{99m}Tc chelate at high specific activity. In the receptor-saturation experiment, the live and dead cells were incubated with 0.2, 2, 10, 20, and 100 μg of Na-HEDP per milliliter of medium for 48 hr before the experiments.

RESULTS

After incubation with ^{99m}Tc-pyrophosphate and ^{99m}Tc-diphosphonate, about 15–20 times more activity was associated with the dead cells than with the live cells (Figs. 1–3). When ^{45}Ca was used along with ^{99m}Tc-labeled pyrophosphate or diphosphonate, the ^{45}Ca activity in the dead cells was only two to three times that in the live cells. No preferential uptake in dead cells was seen with ^{67}Ga-citrate (Fig. 4). The ^{67}Ga uptakes were very similar for both live and dead cells, except that the uptake of ^{67}Ga-citrate by dead cells appeared faster, most probably due to membrane damage.

To study the effect of denaturation of cell components on cellular uptake, live cells were denatured by heating for 10 or 30 min at 50°C. The cells were then incubated with the radiopharmaceuticals in medium. The uptake increased with the duration of heating (Figs. 5 and 6). Under the light microscope, the denatured cells resembled the live cells (dead cells, on the other hand, would show pyknotic nuclei). Live cells adhere to the surface of the culture flask, whereas dead cells slough off the surface and pick up trypan blue. Due to this property of adherence, live cells could be clearly separated from dead cells by a change of medium.

To study the effect of any competitive binding of ^{99m}Tc-HEDP with Ca-HEDP or Na-HEDP, the live and dead cells were pretreated with Na-HEDP. At 200 μg/ml of medium, we found that Na-HEDP was cytotoxic. The cells that died of the toxic effect picked up ^{99m}Tc chelates like the dead cells from plateau-phase culture. Although the uptake of ^{99m}Tc chelates by live cells was not affected, the uptake by dead cells was significantly reduced (Fig. 7).

DISCUSSION

Our data show that the ^{99m}Tc-labeled tracers used for myocardial infarct imaging also localize preferentially in dead cells in tissue culture. We have shown in previous studies that ^{99m}Tc-tetracycline binds to denatured macromolecules of DNA, RNA, and protein (*5*). The structural changes associated with the degeneration of subcellular components in dead cells lead to conformational alterations of the macro-

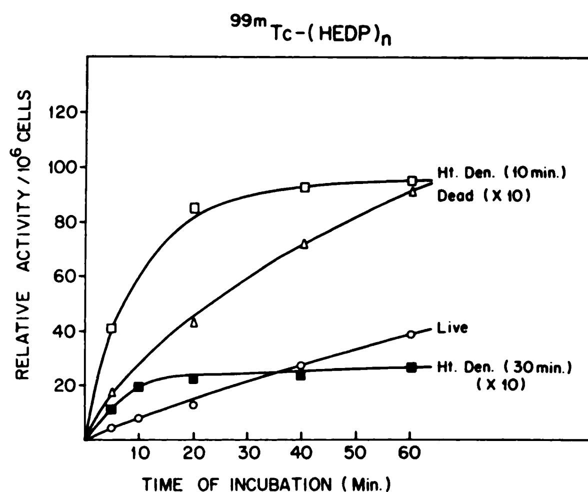

FIG. 5. Effect of heat-denaturation on cellular uptake of ^{99m}Tc-diphosphonate. Each data point for relative uptake of ^{99m}Tc-(HEDP)$_n$ in dead cells and in cells heat-denatured for 30 min should be multiplied by 10 for comparison with relative uptake of live cells. Localization of ^{99m}Tc-(HEDP)$_n$ in dead cells is higher than that in live cells by one order of magnitude.

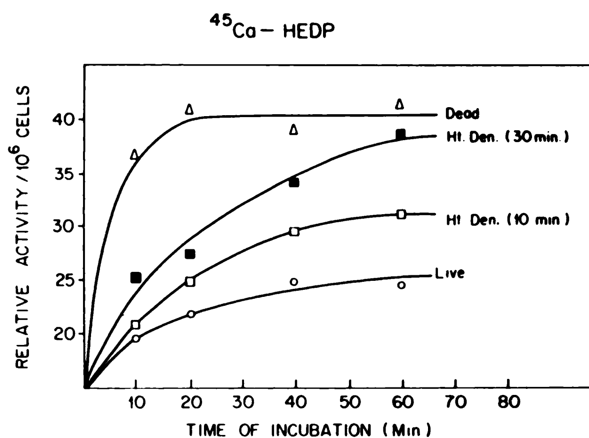

FIG. 6. Effect of heat-denaturation on cellular uptake of ^{45}Ca-diphosphonate.

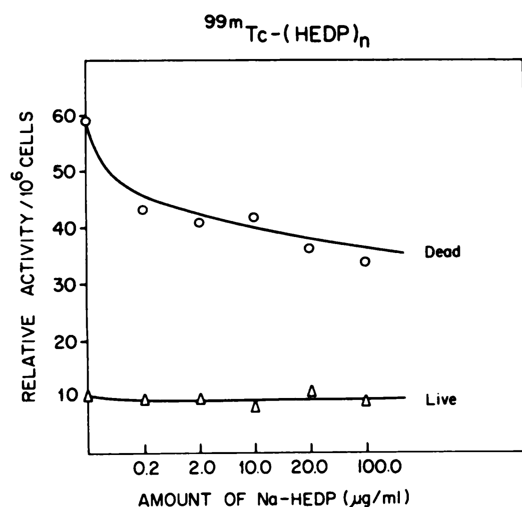

FIG. 7. Effect of preincubation of cells with Na-diphosphonate on uptake of ^{99m}Tc-diphosphonate by live and dead cells.

molecules, allowing the open tertiary structure to bind a variety of metal ions and metal chelates (*5–13*). Because of the similarity of localization of ^{99m}Tc-pyrophosphate and ^{99m}Tc-diphosphonate and the stability of diphosphonate, we used ^{99m}Tc-diphosphonate in most of the experiments. It is useful to have a constant statistically significant number of cells (1,000,000 cells) in the labeling experiments; in this way any effect due to variation in cell numbers can be avoided. The cells die in tissue culture at a definite rate, but the amount of membrane damage and subsequent loss of intracellular components increases after cell death. Hence, we find that dead cells should be harvested within a short time (3–5 days) after the change of medium.

The labeling characteristics of cells for ^{67}Ga-citrate indicate that the mechanism of localization of ^{99m}Tc chelate in dead cells is different from that of ^{67}Ga-citrate. The ^{67}Ga-labeled white blood cells accumulate in the later phase of an acute infarct, when the necrotic cells are removed by phagocytosis (*10*). In canine myocardial infarct, a poor activity ratio of infarcted versus normal myocardium was obtained because only a small fraction of ^{67}Ga binds to the white blood cells and the rest of the activity clears slowly from the circulation.

The subcellular fractions of ^{99m}Tc-diphosphonate-labeled cells (Tables 1 and 2) show that only a small fraction of radioactivity is associated with the nuclei, mitochondria, and microsomes; most of the activity was found in the soluble fraction. A similar distribution is also obtained for ^{45}Ca-HEDP. This distribution is not substantially altered when dead cells are pretreated with Na-HEDP. If the hydroxyapatite in mitochondria was the only site for binding, presaturation should have reduced further the fraction in mitochondria, but no major change in subcellular distribution is observed. Due to the possibility of cross-contamination, a pure fraction of subcellular organelles is difficult to achieve. Better separation could be achieved only at the expense of quantitative distribution.

The kinetics of cellular uptake of ^{45}Ca and ^{99m}Tc chelates are significantly different. Calcium ion forms very weak complexes with pyrophosphate and diphosphonate (the logarithm of the equilibrium constant of Ca-diphosphonate is 2.27), as well as with amino acids and heat-inactivated fetal calf serum. The reduced uptake due to the carrier effect, poor Ca binding with cellular proteins, and the high probability of ligand exchange give rise to a dead-to-live-cell activity ratio of 2; this ratio stays almost constant with time of incubation. We also observed that the receptor sites for ^{99m}Tc-HEDP could be partially blocked with Na-HEDP, either due to the competition of Ca-HEDP or with Na-HEDP itself. In dead cells the ^{99m}Tc uptake is reduced 50% by the presence of 100 μg of Na-HEDP, whereas this drastic change in uptake is not observed in labeling live cells.

When live cells are denatured thermally, their incorporation of ^{99m}Tc-HEDP increases. This uptake depends on the duration of heating and is directly proportional to the degree of denaturation of macromolecules (*12,13*). We have used this effect indirectly to screen infarct-seeking radiopharmaceuticals

TABLE 1. SUBCELLULAR DISTRIBUTION IN ^{99m}Tc-HEDP-LABELED LIVE AND DEAD MAMMALIAN CELLS*

Cell fraction	Live	Dead
Nuclei	21.5 ± 3.5	27.5 ± 3.5
Mitochondria	8.5 ± 1.6	12.3 ± 2.5
Microsomes	2.2 ± 0.4	1.8 ± 0.3
Soluble fraction	67.8 ± 6.5	58.4 ± 5.5

* Data are expressed as percent of bound activity per cell fraction.

TABLE 2. SUBCELLULAR DISTRIBUTION IN ^{45}Ca-HEDP-LABELED LIVE AND DEAD MAMMALIAN CELLS*

Cell fraction	Live	Dead
Nuclei	15.5 ± 2.1	8.5 ± 1.6
Mitochondria	5.5 ± 1.1	6.5 ± 1.3
Microsomes	2.5 ± 0.6	1.8 ± 0.4
Soluble fraction	76.5 ± 8.5	83.2 ± 9.1

* Data are expressed as percent of bound activity per cell fraction.

for localization in heat-damaged myocardium in a rat model. The main drawback of that model is the lack of reproducibility due to the difficulty of obtaining a uniform layer of heat-damaged cells and change in microcirculation. The tissue samples representing damaged myocardium will contain a variable number of normal cells. On the other hand, the necrosis model described in this paper provides a uniform cell population in the live and dead phases. Also, we have observed that the degree of uptake of ^{45}Ca and ^{32}P-pyrophosphate is poor in heat-damaged myocardium. From these different points of view, the cellular necrosis model provides a very useful system for screening tracers for myocardial infarct imaging as well as for studying the mechanism of localization of these agents. Our results indicate that bone-seeking ^{99m}Tc chelates could be used as markers for dead cells in radiation biology and tissue-culture studies, in place of the different dyes now used for staining.

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