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SHORT COMMUNICATION

Intracellular Sequestration of ^{223}Ra by the Iron-Storage Protein Ferritin

Michael J. Atkinson,[1] Marianne T. Spanner, Michael Rosemann, Utz Linzner, Walter A. Müller and Wolfgang Gössner[2]

Institute für Pathologie, GSF-Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstrasse 1, D85764 Neuherberg, Germany

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Incorporation of bone-seeking, α-particle-emitting, heavy-metal radionuclides dramatically increases the incidence of osteosarcoma in humans and experimental animals. The accumulation of these radionuclides within the mineral phase of the bone matrix is believed to result in local irradiation of only those proliferating cells close to the bone surface. We now present evidence for a more general pathway for the irradiation of target cells, mediated through the sequestration of heavy-metal radionuclides by the intracellular iron-storage protein ferritin. *In vitro* studies reveal the transfer of radionuclide from a ^{223}Ra-transferrin complex into immunoprecipitable cytosolic ferritin. *In vivo* studies confirm the co-localization of incorporated ^{224}Ra and cellular iron stores. This pathway would result in the highly localized irradiation of ferritin-containing cells. Since osteoblastic cells express large quantities of a ferritin isoform specialized in long-term metal storage, we suggest that this may represent an unrecognized source of intracellular irradiation by α-particle-emitting radionuclides. Such a local concentration within target cells has implications both for cellular dosimetry and for inferences of track length and target cell populations within the skeleton.

INTRODUCTION

Sarcoma of the bone is a rare malignant disease, affecting less than 1 in 50,000 individuals (*1*). Epidemiological studies have established that bone-seeking α-particle-emitting radionuclides are effective sarcomagenic agents, increasing tumor incidence by up to 1000-fold in exposed individuals (*2, 3*). Bone-forming osteosarcomas are the most prevalent solid tumor, but chondrosarcoma and malignant fibrous histiocytoma (MFH) are also encountered in radiation-exposed cohorts (*4*). Although all of these tumor types originate from the same mesenchymal lineage, the identity of the target cell(s) transformed during osteosarcomagenesis remains unclear. Defined empirically, by following the distribution of the incorporated radionuclides into newly formed bone matrix, they are mature osteoblasts on the bone surface and their immediate precursors situated within 10 μm of the surface (*5*). However, these cells are already committed to osteoblastic differentiation, so their malignant transformation may not give rise to all of the histologically diverse mesenchymal tumor types encountered. The multipotent mesenchymal stem cell population of the bone marrow stroma offers a plausible alternative target (*6*), although here the distance of the stroma from the bone surface may limit exposure to incorporated radionuclides. For this alternative model to function, it would be necessary to have the incorporated radionuclide closer to the target stromal cells.

We have shown previously that the ubiquitously expressed iron-sequestering heteropolymeric protein ferritin is present in osteoblastic cells and have demonstrated that these cells exhibit an active pathway for the uptake of iron from the circulating iron-transporting protein transferrin (*7*). Since both transferrin and ferritin are capable of sequestering a multitude of other metals, including radioactive heavy metals (*8–10*), we postulate that ferritin may be a significant reservoir for radionuclide deposition into both skeletogenic and hematopoietic cells. This suggests that transformation of mesenchymal stem cells by locally deposited intracellular radionuclide may be an additional mechanism to consider in osteosarcomagenesis.

MATERIALS AND METHODS

Radionuclides and Preparation of Transferrin Conjugates

Radium-223 was prepared by chemical separation from ^{227}Ac (*11*) while ^{224}Ra was purchased from Amersham (Braunschweig, Germany). A ^{223}Ra-transferrin complex for uptake studies was generated by co-incubating iron-free rat transferrin (Boehringer, Mannheim, Germany) with a ^{223}Ra-nitrilotriacetic acid chelate (*12*).

[1] Address for correspondence: Institut für Pathologie, GSF-Forschungszentrum, Ingolstädter Landstrasse 1, D85764 Neuherberg, Germany; e-mail atkinson@gsf.de.

[2] Deceased.

Incubation of Osteoblastic Cells with Radiolabeled Transferrin

ROS 17/2.8 rat osteoblastic osteosarcoma cells (*13*) were cultured in Dulbecco's modified Eagle medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and 5% newborn calf serum. ^{223}Ra-transferrin (100 kBq/culture) was added to subconfluent cultures maintained in 250-ml flasks for 48 h. Cells were rinsed twice with ice-cold phosphate-buffered saline and lysed (*7*).

Biosynthetic Labeling of Ferritin with ^{35}S-Methionine

In vitro labeling of ferritin with ^{35}S-methionine was performed by culturing semi-confluent exponentially growing ROS 17/2.8 cells for 3 h with methionine-free DMEM plus 37 MBq ^{35}S-methionine (Amersham, Braunschweig, Germany). After a chase with unlabeled methionine, the cell monolayers were washed with PBS and lysed (*7*).

Immunoprecipitation of Ferritin

Non-ferritin proteins were partially removed by heat denaturation of cell lysates at 60°C followed by a preclearing incubation with agarose-coupled goat anti-rabbit secondary antibody (Sigma, Deisenhofen, Germany). After removal of the solid-phase secondary antibody, the cellular ferritin was immunoprecipitated by adding 20 μg affinity-purified rabbit anti-ferritin IgG (Dakopatts, Hamburg, Germany) and 25 μg of secondary antibody to 100 μl cell lysate. For control immunoprecipitates, 20 μg of non-immune rabbit IgG was substituted for the primary antibody. After extensive washing, immunoprecipitates were resolved by non-denaturing polyacrylamide gel electrophoresis. The ^{35}S- or ^{223}Ra-labeled proteins were revealed by autoradiography.

Intracellular Detection of Radium within Osteosarcoma Cells

Monolayer cultures of ROS 17/2.8 cells exposed to ^{223}Ra-transferrin as above were grown to semi-confluence on microscope slides, fixed in 1% paraformaldehyde, and dehydrated in ethanol. Slides were coated with NTB-2 autoradiographic emulsion (Eastman Kodak, Rochester, NY) diluted 1:2 in deionized water and air-dried for storage. After 21 days, slides were developed, fixed in 30% sodium thiosulfate, and dried.

Co-localization of ^{224}Ra and Inorganic Iron In Vivo

Four-week-old female NMRI mice obtained from the GSF breeding colony were injected with ^{224}Ra as radium chloride (18.5 kBq/kg body weight) and killed humanely after 30 h. Animals were housed according to national laboratory animal welfare regulations, and studies were performed under federal license. Spleen tissue, a rich source of ferritin, was removed, and formalin-fixed, paraffin-embedded tissue sections were coated with NTB emulsion for autoradiography. After development, iron deposits were revealed by the Prussian Blue (ferric ferrocyanide) histochemical stain.

RESULTS

Co-localization of ^{223}Ra with Immunoprecipitated Ferritin from Osteoblast Cell Cultures

Autoradiographic imaging of the protein immunoprecipitation by the anti-ferritin antibody from ROS 17/2.8 osteoblastic cells preincubated with ^{223}Ra-labeled transferrin revealed a single high-molecular-weight band. This ^{223}Ra-labeled protein co-migrated with both a native ferritin marker protein and the ^{35}S-methionine-labeled protein precipitated with the same anti-ferritin antibody (Fig. 1A). This confirms that radium had been transferred from extracellular transferrin to intracellular ferritin.

Autoradiography Detection Reveals α-Particle Tracks Associated with Cytoplasm

To confirm that the source of ^{223}Ra radiation was cytosolic ferritin, we examined autoradiographic images of the *in vitro* deposition of radium within ROS 17/2.8 cell cultures. After preincubation with ^{223}Ra-labeled transferrin (as above), autoradiography revealed that the great majority of the α-particle tracks overlap the cells (Fig. 1B).

Co-localization of ^{224}Ra and Iron in Mouse Spleen

To demonstrate that ferritin is able to incorporate radium *in vivo*, we followed the fate of ^{224}Ra injected intravenously in mice. Autoradiographic examination of the spleen, the second most prevalent site of ^{224}Ra deposition (*14*), revealed concentrations of α-particle tracks that appear to fan out from areas of the tissue that stain intensely with the ferric ferrocyanide reaction product (Fig. 1C). This co-localization of ^{224}Ra with iron supports the argument for ferritin-mediated sequestration of heavy-metal radionuclides within tissues.

DISCUSSION

We have demonstrated cellular incorporation of ^{223}Ra from a ^{223}Ra-transferrin complex in osteoblast-like cells *in vitro*. The internalized radionuclide was shown to be deposited within a heat-stable protein with a molecular weight of 450 kDa that was immunologically identified as the cytosolic iron-binding protein ferritin. Such an association between heavy-metal radionuclides and cellular ferritin has long been suggested by the co-localization of plutonium and hemosiderin (*15*) as well as by the co-purification of ferritin with plutonium in liver tissue (*8*) and by the ability of ferritin to capture uranium in a cell-free system (*9*).

Cytosolic ferritin is able to enzymatically oxidize reactive Fe(II) and sequester the Fe(III) product within a nanocrystal of inorganic iron embedded within the central pore of the ferritin holoprotein (*16*). However, ferritin appears to be unable to discriminate between iron and other metals, leading to suggestions that ferritin may initially have evolved as a general metal scavenger molecule (*10*). The mechanism by which these non-ferrous metals become retained in the metal core is unclear, but it may be through an inorganic process (*17*).

The biological relevance of the sequestration of radium and other bone-seeking metal radionuclides by the osteoblast lies in the special properties of the bone isoform of ferritin. Ferritin holoproteins are composed of a 24-subunit mixture of heavy (H) and light (L) ferritin chains, produced by separate genes. Osteoblast ferritin is highly enriched for the L isoform, which is primarily responsible for the long-term storage of iron, having a much lower level of the ferroreductase catalytic activity (*7*), this being essential for the rapid Fe(II) turnover that characterizes the H-isoform-rich ferritins (*18*). The production of an L-isoform-enriched

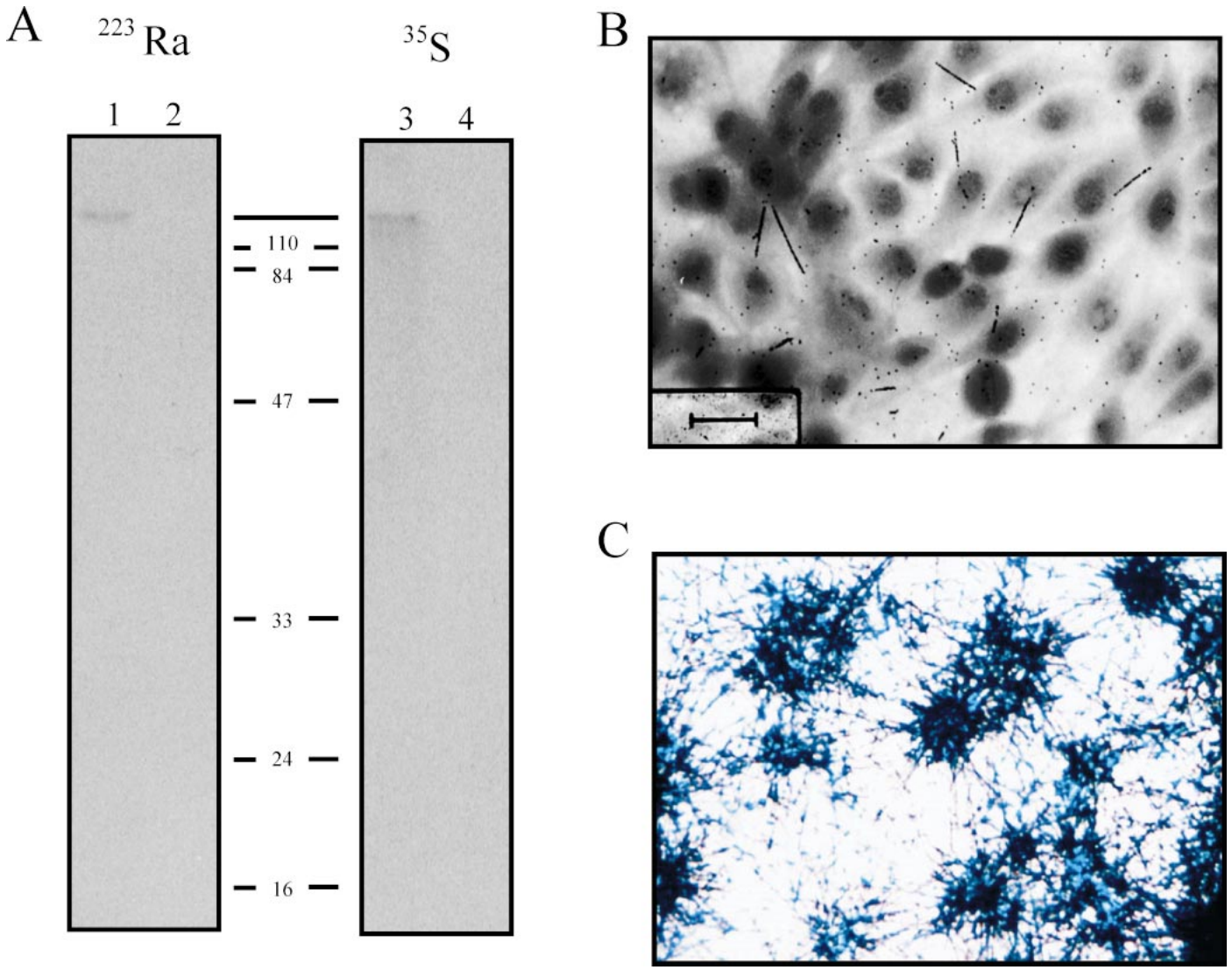

FIG. 1. Panel A: Autoradiographic images of proteins immunoprecipitated from ROS 17/2.8 rat osteosarcoma cells incubated with ^{223}Ra-transferrin or ^{35}S-methionine. After removal of heat-labile proteins, the cell lysate was incubated with an anti-ferritin antiserum, which was subsequently precipitated by a solid-phase secondary antibody. Non-denatured polyacrylamide gel electrophoresis was used to separate proteins. Horizontal bars indicate the position of molecular weight markers (kDa). The upper solid bar corresponds to native ferritin protein. Lanes 1 and 3 were immunoprecipitated with anti-ferritin antibody, lanes 2 and 4 with a non-immune antibody. Panel B: Autoradiographic image of ROS 17/2.8 cells incubated with ^{223}Ra-transferrin. The cell monolayer was fixed and coated with emulsion to reveal α-particle tracks. Note that the track origins and/or termini overlap cytoplasm. Scale bar is 10 μm. Panel C: Autoradiographic image of a tissue section taken from the spleen of a ^{224}Ra-injected mouse. Iron deposits (hemosiderin) within the red pulp of the spleen were revealed by histochemical staining (Prussian Blue) and are visible as an intense blue color. Note the apparent source of ^{224}Ra radiation is co-localized with the blue staining.

ferritin, such as that found in osteoblasts, is hence a characteristic of tissues with long-term high-capacity storage of iron such as liver and spleen (*19*). Thus it appears that bone cells have an inherent capacity for the long-term storage of iron and, by default, other metals in cytosolic ferritin.

Osteoblastic cells undergo terminal differentiation or cell death by apoptosis after a life span approximating 1 month (*20*). While this may exceed the half-life of some potential ferritin-deposited radionuclides, others have much greater half-lives. The fate of ferritin and sequestered metals during apoptosis is unknown; however, release and recapture within the local environment is one alternative fate that would lead to retention of the radionuclide at or near the bone surface.

The closeness of surface-deposited radium to bone-lining osteoblast and preosteoblast cells, coupled with the short track length of α particles, has been used to explain the osteosarcomagenic potential of the radionuclide. The alternative histion model, recently proposed to explain the different malignant cell types encountered after radium deposition into the skeleton, suggests that the target cells are multi- and pluripotent mesenchymal progenitor cells located within the marrow stroma and not at the bone surface (*6*). However, following this model, surface-deposited ra-

dionuclide would not be able to directly irradiate the stroma under conditions of local cell proliferation where stroma and bone surface are separated by layers of osteoblastic precursors and maturing osteoblasts. In areas of low bone turnover, where only lining cells are present, the distance of target cells would be reduced.

The maximal distance of ferritin from the bone surface would be one or two osteoblast/preosteoblast layers deep, thereby increasing the range of α particles and bringing the radionuclide much closer to the target hematopoietic and skeletogenic precursors in the stroma. Indeed, it is not inconceivable that the target cells themselves could sequester radium. Such ferritin-mediated storage may be relevant in determining the risk of late effects from therapeutically applied radium (*21*, *22*).

Alterations in cellular storage capacity may influence the risk of radium-induced osteosarcoma. The level of cellular iron storage is directly modulated by the availability of Fe(II), which induces *de novo* synthesis of both transferrin and ferritin. Thus nutritional status and genetic variability may indirectly influence radionuclide deposition rates, leading to interindividual variation in uptake and storage (*23*–*25*).

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REFERENCES

1. D. M. Homa, M. R. Sowers and A. G. Schwartz, Incidence and survival rates of children and young adults with osteogenic sarcoma. *Cancer* **67**, 2219–2223 (1991).
2. H. S. Martland and R. E. Humphries, Osteogenic sarcoma in dial painters using luminous paint. *Arch. Pathol.* **7**, 406 (1929).
3. C. Mays and H. Spiess, Bone sarcomas in patients given ^{224}Ra. In *Radiation Carcinogenesis: Epidemiology and Biological Significance* (J. B. Fraumeni, Ed.), pp 241–252. Raven Press, New York, 1982.
4. W. Gössner, Pathology of radiation-induced bone tumors. *Leuk. Res.* **10**, 897–904 (1986).
5. A. L. Austin, M. Ellender, J. W. Haines, J. D. Harrison and B. I. Lord, Microdistribution and localized dosimetry of the alpha-emitting radionuclides ^{239}Pu, ^{241}Am and ^{233}U in mouse femoral shaft. *Int. J. Radiat. Biol.* **76**, 101–111 (2000).
6. W. Gössner, Target cells in internal dosimetry. *Radiat. Prot. Dosim.* **105**, 39–42 (2003).
7. M. Spanner, K. Weber, B. Lanske, A. Ihbe, H. Siggelkow, H. Schütze and M. J. Atkinson, The iron-binding protein ferritin is expressed in cells of the osteoblastic lineage *in vitro* and *in vivo*. *Bone*. **17**, 161–165 (1995).
8. G. Boocock, C. Danpure, D. Popplewell and D. Taylor, The subcellular distribution of plutonium in rat liver. *Radiat. Res.* **42**, 381–396 (1970).
9. J. Hainfeld, Uranium-loaded apoferritin with antibodies attached: Molecular design for uranium neutron-capture therapy. *Proc. Natl. Acad. Sci. USA* **89**, 11064–11068 (1989).
10. D. J. Price and J. G. Joshi, Ferritin binding of beryllium and other divalent metal ions. *J. Biol. Chem.* **258**, 10873–10880 (1983).
11. W. A. Müller, Präparative Arbeiten über ^{227}Ac und seine Folgeprodukte. *Radiochim. Acta* **9**, 181–186 (1968).
12. M. Awai, M. Narasaki, Y. Yamanoi and S. Seno, Induction of diabetes in animals by parenteral administration of ferric nitrilotriacetate. *Am. J. Pathol.* **95**, 663–673 (1979).
13. R. J. Majeska, S. B. Rodan and G. A. Rodan, Maintenance of parathyroid hormone response in clonal rat osteosarcoma lines. *Exp. Cell. Res.* **111**, 465–468 (1978).
14. W. A. Müller, Studies on the short-lived internal alpha emitters in mice and rats Part 1. ^{224}Ra. *Int. J. Radiat. Biol.* **20**, 27–38 (1971).
15. J. Arnold and W. Jee, Bone growth and osteoclastic activity as indicated by radioautographic distribution of plutonium. *Am. J. Anat.* **101**, 367–417 (1957).
16. N. Chasteen and P. Harrison, Mineralisation in ferritin; An efficient means of iron storage. *J. Struct. Biol.* **126**, 182–194 (1999).
17. J. Lack, S. Chaudhuri, S. Kelly, K. Kemner, S. O'Connor and J. Coates, Immobilization of radionuclides and heavy metals through anaerobic bioxidation of Fe(II). *Appl. Environ. Microbiol.* **68**, 2704–2710 (2002).
18. P. Arioso and S. Levi, Ferritin, iron homeostasis, and oxidative damage. *Free Radic. Biol. Med.* **33**, 457–463 (2002).
19. P. Harrison and P. Arosio, Ferritins: Molecular properties iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1275**, 161–203 (1996).
20. M. Owen, Lineage of osteogenic cells and their relationship to the stromal system. In *Bone and Mineral Research* (W. A. Peck, Ed.), pp. 1–16. Elsevier, New York, 1985.
21. G. Henriksen, K. Breistol, O. S. Bruland, O. Fodstad and R. H. Larsen, Significant antitumor effect from bone-seeking, alpha-particle-emitting ^{223}Ra demonstrated in an experimental skeletal metastases model. *Cancer Res.* **62**, 3120–3125 (2002).
22. C. Tiepolt, T. Gruning and W-G. Franke, Renaissance of ^{224}Ra for the treatment of ankylosing spondylitis: Clinical experiences. *Nucl. Med. Commun.* **23**, 61–66 (2002).
23. R. C. Lindenschmidt, L. E. Sendelbach, H. P. Witschi, D. J. Price, J. Fleming and J. G. Joshi, Ferritin and *in vitro* beryllium toxicity. *Toxicol. Appl. Pharmacol.* **82**, 344–350 (1986).
24. S. Satarug, P. Ujjin, Y. Vanavanitkun, J. R. Baker and M. R. Moore, Influence of body iron store status and cigarette smoking on cadmium body burden of healthy Thai women and men. *Toxicol. Lett.* **148**, 177–185 (2004).
25. J. W. Haines and N. D. Priest, The release of transportable plutonium from macrophages: The effects of dietary iron. In *Metals in Bone* (N. Priest, Ed.), pp. 71–73. MTP press, Lancaster, 1984.