A Morphological Study of Ferritin Synthesis in Macrophages with Ingested Ferric Hydroxide-Potassium Polyvinyl Sulfate Complexes

Toshiro Ono¹, Tadashi Tsujii² and Satimaru Seno¹

¹Division of Pathology, and ²Division of Ultrastructure Research, Shigei Medical Research Institute, 2117 Yamada, Okayama, 701-02, Japan

ABSTRACT. A ferric hydroxide-polyvinyl sulfate colloidal solution (Fe-PVS), prepared by mixing potassium polyvinyl sulfate (PVSK) and ferric hydroxide colloidal solution was used to study ferritin synthesis in rat peritoneal macrophages. The colloidal particles had spherical electron opaque ferric hydroxide cores with diameters of about 250 nm surrounded by radially arranged fibrous PVS molecules. They also had strong negative electric charges.

Fe-PVS particles injected into the peritoneal cavity were taken up by the macrophages then disintegrated rapidly. In the phagolysosomes the electron opaque ferric hydroxide cores of Fe-PVS were denuded of their PVS frames then decomposed into small 5–6 nm granules 24 to 48 h after injection. These small granules were released from the lysosomes into the hyaloplasm and the myelin figures were found in the lysosomal vacuoles. No reaccumulation of granules in lysosomes was found even 3 months later.

The intracellular distribution of ferritin in macrophages demonstrated by the immunocytochemical method showed a pattern similar to that of the small granules formed by the disintegration of Fe-PVS. This means that in rat peritoneal macrophages that contain ingested Fe-PVS particles ferritin first is synthesized in phagolysosomes by the ferric hydroxide cores that conjugate with apoferritin or protein subunits then they are dispersed into the cytoplasm. Two possible pathways for the biosynthesis of ferritin are discussed.

Ferritin is a ferric hydroxide-apoferritin complex that is widely distributed in animal bodies and serves as a storehouse for iron (13, 21). Heavy deposition of ferritin is induced easily in animals by injecting them with and excess of iron compounds (12, 19, 20). Thus, the process of ferritin formation can be analyzed by using animal cells loaded with iron.

Recent studies of liver cells and hepatocytes given iron compounds *in vivo* as well as *in vitro* have greatly expanded our knowledge of ferritin synthesis.

It is generally believed that apoferritin is synthesized mainly in the cytoplasmic matrix by polysomes and that ferritin is formed there from the apoferritin by the incorporation of Fe(II) (6), after which the synthesized ferritin molecules accumulate in autophagosomes. Recently, it has been demonstrated that macrophages and

Abbreviations used: Fe-PVS, ferric hydroxide-potassium polyvinyl sulfate complex; PVSK, potassium polyvinyl sulfate; PBS, phosphate buffered saline; ER, endoplasmic reticulum.

Kupffer cells in rat liver synthesize ferritin in the same way as do liver parenchymal cells (4).

Whether these two types of cells that have completely different functions have the same pathway for ferritin synthesis is not clear; macrophages have marked phagocytic activity and contain the basic proteins and enzymes that digest phagocytized materials. The iron compounds taken into the phagosomes of the macrophages inevitably are exposed to these active substances which hepatocytes lack. Macrophages take up gross particles of iron hydroxide colloidal particles, red cells and hemoglobin-haptoglobin complexes and use their iron as for ferritin synthesis. Liver cells take up only specific iron compounds such as transferrin which is taken up by receptors on the cell surface (1). Therefore, there should be some difference in the pathway by which ingested iron compounds are synthesized into ferritin between macrophages and hepatocytes, because iron compounds can be taken up by macrophages but not by hepatocytes.

The ferritin molecule is visible by electron microscopy, and apoferritin can be detected by immunocytochemistry. Therefore, the synthetic pathway in macrophages can be analyzed morphologically if we use visible colloidal iron particles that are taken up specifically by macrophages and used the source of iron for ferritin synthesis.

Ferric hydroxide-polyvinyl sulfate colloidal particles (Fe-PVS) are an excellent tool for this analysis. They have a spherical and electron dense iron hydroxide core of about 250 nm that is surrounded by PVS molecules. They also give a strong positive Prussian blue reaction which makes them visible with electron microscopy as well as under light microscopy. In addition, these particles are taken up preferentially by macrophages and disintegrate rapidly into minute iron hydroxide particles in the phagolysosomes of rat peritoneal macrophages (16). These particles are taken up specifically by macrophages, but by no other somatic cells including hepatocytes.

We here show that rat peritoneal macrophages take up Fe-PVS particles and synthesize ferritin in the phagolysosomes very rapidly then discharge the ferritin into the cytoplasmic matrix with, or without, lysis of the limiting membrane. These ferritin particles that are dispersed diffusely into the hyaloplasm never reaccumulate in the autophagolysosomes.

MATERIALS AND METHODS

Preparation of Fe-PVS. The colloidal solution of Fe-PVS was prepared by adding 1 volume of ferric hydroxide colloidal solution, 0.8 mg Fe/ml to 10 volumes of 2.5×10^{-3} N potassium polyvinyl sulfate (PVSK) drop by drop with gentle stirring. The pH of the solution was adjusted to 7.3 by adding 1×10^{-2} M sodium bicarbonate, after which it was made isotonic by adding glucose then filtered through a 450 nm filter. The final concentration of iron was 0.044 mg Fe/ml. The colloidal iron solution was prepared just before use. The PVSK used for colloid titration (15, 24) was obtained from Wako Pure Chemical Indust. Ltd., Osaka.

Animals. Thirty adult male albino rats of the Wistar strain that weighed 250–300 g were used. They were divided into 8 groups, 3 animals each in groups 1 to 7 and 9 animals in group 8.

Intraperitoneal injection of Fe-PVS. Three milliliters of Fe-PVS solution was injected into the peritoneal cavity of each animal in groups 1 through 7. The animals belonging to

group 8 served as controls; each received one intraperitoneal injection of 3 ml of 2.5×10^{-3} N PVSK. Ascites cells were collected from animals in groups 1 through 6 at 1, 5, 24, 48, 120, and 144 h, respectively after Fe-PVS injection. In animals belonging to group 7, ascites cells were taken 3 months after the Fe-PVS injection. Cells were obtained from 9 control animals, 3 animals at a time, 24, 72, and 144 h after PVSK injection. The ascites fluid obtained from the 3 animals in each group was combined and mixed then used in the experiments. Each experiment was repeated 3 times.

Preparation of antibody to ferritin. Rabbit antiserum to ferritin was prepared with an injection of rat liver ferritin emulsified with complete Freund's adjuvant. Rabbit antibody specific to ferritin was obtained and purified by affinity chromatography as follows: Ferritin was covalently linked to BrCN-activated Sepharose 4B (0.4 mg protein/ml Sepharose) (11). The antiserum to ferritin was added to the Sepharose 4B column, which then was washed with PBS until the eluate showed no absorbance at 280 nm.

The bound antibodies were eluted with 2 M NaSCN. The effluent was collected and dialyzed exhaustively against PBS. The immunodiffusion test was done by the Ouchterlony double diffusion method. Rat liver ferritin was given by Dr. R. Matsuura (Okayama University Medical School).

Light microscopy of ascites smears. One droplet of the ascites fluid was smeared on a slide and used for light microscopy. The smear was fixed in methanol then treated with potassium ferrocyanide for the Prussian blue reaction, after which it was post-stained with hematoxylin.

Electron microscopy of peritoneal macrophages. Cell pellets obtained by centrifuging the ascites cells for 10 min at 900 rpm, were used for electron microscopy. The sedimented cells were fixed in 2% glutaraldehyde for 1 h at 4°C. After fixation these cells were suspended in 5×10^{-2} M sodium cacodylate buffer at pH 7.3 then washed with the same solution. The cells then were post-fixed in 2% OsO₄ for 1 h after which they were dehydrated through graded ethanols to acetone then embedded in TAAB resin. Thick sections of about 2 μ m and subsequent thin sections of silver color were cut with a Reichert OmU-3 ultramicrotome. The thick sections were used for light microscopy and the thin ones for electron microscopy. The thin sections were observed in a Hitachi electron microscope, H-700, with or without staining by uranyl acetate and lead citrate.

Identification of ferritin by immunofluorescence. The indirect immunofluorescence method (14) was used. Smears of ascites cells fixed with methanol were rehydrated in PBS for 1 min. These cells then were incubated for 30 min at room temperature with rabbit anti-rat ferritin antibody that had been purified by the method described above. After incubation the cells were washed with PBS and exposed to goat anti-rabbit fluorescein isothiocyanate-conjugated IgG (1 : 50 dilution in PBS, Cappel Laboratories Inc., Cochranville, Pa. U.S.A.) for 30 min then washed and observed under a fluorescence microscope. For the control, cells were incubated with normal rabbit serum in place of rabbit anti-ferritin antibody then stained with goat fluorescein-IgG.

RESULTS

Characteristics of Fe-PVS particles. Colloidal particles of Fe-PVS prepared by the method described had strong negative electric charges, 0.6 mA/cm for 30 min, when electrophoresed on cellulose acetate membranes (22, 23). Particles gave a positive Prussian blue reaction. Electron microscopy showed them as electron dense spherical particles about 250 nm in diameter that were framed by radially arranged fibrous PVS molecules (Fig. 1).

Fig. 1. EM photograph of Fe-PVS particles injected into the peritoneal cavity of a rat. Stain: uranyl acetate and lead citrate. $\times 50,000$.

Fig. 2. Ouchterlony immunodiffusion test made with rat ferritin and purified antibody. A-F: affinity purified anti-rat ferritin antibody, F: rat liver ferritin, NS: normal rabbit serum.

Fig. 3. LM photographs of rat ascites taken 24 (a) and 144 h (b) after the i.p. injection of Fe-PVS. Cells were smeared and fixed then stained by the Prussian blue reaction and post-stained with hematoxylin. Fe-PVS showing a positive Prussian blue reaction appears as dark granules in the cytoplasm. (a) Peritoneal macrophages have Fe-PVS (arrows), but granulocytes, lymphocytes and mast cells do not. (b) Three peritoneal macrophages. Most granules in the cytoplasm had disappeared after 144 h. $\times 1,000$.

Specificity of the antibody. The specificity of the purified antibody for ferritin was analyzed by immunodiffusion. A single precipitin line was formed between rat ferritin and the purified antibody (Fig. 2). No reaction took place between normal rabbit serum and the ferritin.

Light microscopy of ascites smears. Ascites cells collected 1 h after an i.p. injection of Fe-PVS had peritoneal macrophages that actively took up Fe-PVS as shown by the Prussian blue reaction. Most macrophages had several blue granules in their cytoplasm. Also, they had homogenous blue masses of Fe-PVS adhering to their cell surfaces. Macrophages taken 5 h after the Fe-PVS injection presented nearly the same picture, but the Fe-PVS masses on their cell surfaces were much reduced and the number of intracellular blue granules had increased. Twenty four hours after injection macrophages had lost the Fe-PVS on their surfaces and the intracellular blue granules tended to be large agglomerates (Fig. 3a). These agglomerates seemed to become smaller, but were more dense 48 h after the Fe-PVS injection. After 120 h the blue granules had grown much larger, but there were fewer of them. In addition, they had cytoplasmic vacuoles and gave a negative Prussian blue reaction. After 144 h, the number of macrophages with cytoplasmic vacuoles had increased, most were filled with Prussian blue negative vacuoles and a small number of blue granules (Fig. 3b). After 3 months no macrophage that gave a positive Prussian blue reaction was found.

Electron microscopy.

1. *Electron microscopy of sections stained with uranyl acetate and lead citrate.* Observations of ascites cells on stained sections at specific intervals after Fe-PVS injection clearly showed the process of Fe-PVS uptake and decomposition by macrophages. Macrophages taken 1 h after the Fe-PVS injection had a number of Fe-PVS particles in their phagocytic vesicles and some particles adhering to their cell surfaces (Fig. 4a). Particles also were found in the phagosomes of macrophages sampled 5 h after Fe-PVS injection.

In some phagosomes the interparticular spaces appeared dark. These are the phagolysosomes formed by the fusion of phagocytic vacuoles with lysosomes (Fig. 4b). Twenty four hours after the Fe-PVS injection the number of phagolysosomes had increased. Each was smaller but its contents were denser. At this stage some myelin figures appeared in the phagolysosomes (Fig. 4c).

Forty eight hours after the Fe-PVS injection macrophages had large phagolysosomes containing Fe-PVS particles in various stages of disintegration and some myelin figures. In some phagolysosomes the limiting membrane became faint and its contents scanty, but there were some myelin figures and minute electron dense granules present (Fig. 4d). One hundred forty four hours after the Fe-PVS injection macrophages had a number of phagolysosomes, most with few contents, some had myelin figures with a few minute electron dense granules (Fig. 4e).

These results show that the Fe-PVS particles were attacked and decomposed by lysosomal enzymes in the phagolysosomes. Macrophages from controls given PVS-K also had a large number of vacuoles. They also had myelin figures but no electron dense minute particles. This is evidence that Fe-PVS particles are decomposed in the phagolysosomes, the ferric hydroxide core being denuded of PVS, decomposed into minute granules then dispersed into the hyaloplasm.

2. *Observations of unstained sections.* Detailed information about the dis-

integration of the ferric hydroxide core of Fe-PVS was obtained by observing unstained sections in which minute electron dense granules that were dispersed diffusely in cytoplasm could be seen clearly. Decomposition of the Fe-PVS particles took place in the phagolysosomes of the macrophages. Macrophages collected 5 h after the Fe-PVS injection had electron dense homogeneous ferric hydroxide cores of Fe-PVS in their phagolysosomes that were being disintegrated into minute granules 5–6 nm in diameter. Some of these granules also were found in the cytoplasmic matrix (Fig. 5a), but they were never present in the cytoplasm of macrophages from controls injected with PVS-K and Hanks' solution.

A more advanced stage of the decomposition of the Fe-cores was seen in macrophages collected 48 h after the Fe-PVS injection. Small granules were well separated and were dispersed in the cytoplasmic matrix, except in the internal spaces of the mitochondria and endoplasmic reticulum (ER). The limiting membrane of the phagolysosomes often had disappeared (Fig. 5b). High magnification of the granules showed their individual shapes were like the iron-core of ferritin (Fig. 5b, inset).

At 120 and 144 h after the Fe-PVS injection, ferric hydroxide cores in advanced stages of decomposition were observed. The outline of the Fe-PVS core was very amorphous, and dense distribution of granules in the hyaloplasm was common. Numerous vacant vacuoles had been formed by the discharge of granules, but aggregates occasionally were present in the vacuoles (Fig. 6a).

Three months after the Fe-PVS injection, macrophages were similar to those collected 144 h after injection, but phagolysosomes were few and most of the small

Fig. 4. EM photographs of peritoneal macrophages taken 1 (a), 5 (b), 24 (c), 48 (d) and 144 h (e) after the i.p. injection of Fe-PVS. Cells were stained with uranyl acetate and lead citrate. A number of Fe-PVS particles with electron dense cores are present in the phagocytic vesicles (a, b, c and d). (a) Fe-PVS particles in the phagosomes of the macrophages have nearly the same configuration as those found in intercellular spaces. (b) After fusion of the phagosomes with lysosomes (arrowhead) the PVS molecules surrounding the Fe cores became amorphous and denser (arrows). (c) Ferric hydroxide cores were denuded of their PVS coats when myelin figures formed (arrows). Note that neighboring lymphocytes have not ingested Fe-PVS particles (a and c). (d) Macrophage with decomposing Fe-PVS. The decapsulation of Fe-PVS particles at advanced stages is shown by the formation of distinct myelin figures in the phagolysosomes. (e) Macrophage with myelin figures (arrows) and some electron dense gross and minute particles in phagolysosomes. No Fe-PVS particles with original figures are present. There are few phagolysosomes 144 h after the Fe-PVS injection. a–c, $\times 11,000$; d, $\times 16,500$; e, $\times 15,000$.

Fig. 5. EM photographs of parts of the cytoplasm of peritoneal macrophages taken 5 (a) and 48 h (b) after the i.p. injection of Fe-PVS. These unstained sections clearly show the early stage of decomposition of the ferric hydroxide cores into small granules about 5–6 nm in diameter. Some granules are dispersed in the hyaloplasm (arrows), evidence that decomposition already had taken place 5 h after the Fe-PVS injection (a). Decomposition of ferric hydroxide cores proceeds sequentially (b). High magnification of minute particles in the hyaloplasm (inset in b). Unstained section. a and b, $\times 60,000$; inset in b, $\times 250,000$.

Fig. 6. EM photographs of parts of the cytoplasm of peritoneal macrophages taken 144 h (a) and 3 months (b) after the i.p. injection of Fe-PVS. The configuration of the lysosomal membrane has become faint (arrows) and ferritin particles are freely dispersed in the hyaloplasm. A few aggregated granules are present in some lysosomes (arrowhead) at 144 h of the experiment (a). Three months after the injection, ferritin particles are dense in only a few lysosomes, most granules have dispersed into the hyaloplasm (b). Particles in the lysosomes are residual. Most lysosomes are empty. No specific structures that would support the idea of the reaccumulation of granules in lysosomes are present. Unstained section. $\times 50,000$.

Fig. 7. Indirect immunofluorescence micrographs of ferritin distribution in macrophages taken 24 (a) and 120 h (b) after the i.p. injection of Fe-PVS. (a) Fluorescence is present in the cytoplasmic vacuoles in gross masses. (b) Fluorescence is diffuse in the cytoplasm, but is more intense than in a. No fluorescence is present in the cytoplasmic vacuoles (arrows). $\times 1,200$.

granules were found in the cytoplasmic matrix (Fig. 6b). Some vacuoles had granules dispersed diffusely but no microscopy showed any reaccumulation of granules in the autophagic vacuoles.

Immunofluorescence staining of ferritin in macrophages. Macrophages that had ingested Fe-PVS particles gave specific immunofluorescence for ferritin. In the period 24 to 48 h after the Fe-PVS injection macrophages stained with rabbit anti-rat ferritin antibody and goat anti-rabbit FITC IgG showed clear fluorescence on the vacuoles in their cytoplasm (Fig. 7a). The cytoplasmic matrix however, had hardly any fluorescence at this stage.

After 120 to 144 h macrophages had strong but diffuse fluorescence in the cytoplasm, indicating that ferritin was scattered diffusely throughout the cytoplasm (Fig. 7b). At this stage there was no vacuole fluorescence, evidence that ferritin had moved out of the vacuoles. Our results clearly show that ferritin first is synthesized in the phagolysosomes then is dispersed into the cytoplasmic matrix.

DISCUSSION

Our results clearly demonstrate that Fe-PVS particles taken into the phagolysosomes of the macrophages were decomposed very quickly. Disintegration started as early as 5 h after the Fe-PVS injection; it proceeded rapidly with lapse of time. Granules were dispersed into the cytoplasmic matrix with, or without, lysis of the limiting membrane of the phagolysosome. Finally, nearly all these granules were discharged from the phagolysosomes and were dispersed in dense patterns in the cytoplasmic matrix, where they remained for more than 3 months, no reaccumulation taking place in the cytoplasmic vacuoles. These newly formed and small electron dense granules are very similar in size and morphology to the iron core of ferritin.

Immunofluorescence staining for ferritin showed that these granules actually are ferritin particles. This means that the Fe-PVS particles phagocitized by macrophages were decomposed rapidly to minute granules in the phagolysosomes then utilized for ferritin synthesis. Our findings are clear evidence that in macrophages ferritin first is formed in the phagolysosomes after which it is dispersed into the cytoplasmic matrix.

Our findings and conclusions do not agree with the current belief that apoferritin is synthesized mainly in the cytoplasmic matrix by polyribosomes (7, 18) and that ferritin first is formed in the cytoplasm by the incorporation of Fe(II) into the apoferritin molecules (6) then is translocated to the endolysosomes (12, 26).

This biosynthesis of ferritin has been demonstrated by loading liver hepatocytes with an iron compound such as iron-dextran. Richter and his co-investigators stressed that approximately 85% of the apoferritin is synthesized by free polyribosomes and the remainder by membrane-attached polyribosomes (9, 10, 17), and that finally ferritin molecules synthesized in the cytoplasmic matrix are accumulated in the endolysosomes through an autophagic mechanism (5, 8, 25). The accumulation of ferritin molecules, which had been widely distributed in the cytoplasmic matrix, in endolysosomes must be an enormous, free energy-consuming reaction.

Although apoferritin molecules synthesized by membrane-attached polyribosomes reach the lysosomes by direct channeling through the ER and the Golgi apparatus (3, 25), Trump *et al.* showed that ferritin also is synthesized in hepatocytes by membrane-attached polyribosomes, then is released into the lumen of the rough ER and transported to lysosomes through the Golgi apparatus from the fact that they found ferritin molecules occasionally within the lumen of the rough ER and the Golgi cisternae (25). They also suggested that apoferritin molecules synthesized in the membrane-attached polyribosomes move to the Golgi apparatus then to the lysosomes where they incorporate iron to form ferritin (2, 25).

Our observations support the view that apoferritin, or its protein subunits, is synthesized by the membrane-attached polyribosomes and enters the lysosomes where it is synthesized to ferritin. We could not, however, obtain evidence that the 5–6 nm granules are decomposed into smaller ones and, finally, to invisible ferrous ion [Fe(II)] that then is incorporated into preexisting apoferritin molecules. These granules never disappeared, even briefly; they maintained the same size and morphology as the iron core of ferritin. The granules seemed to take up apoferritin molecules, or their subunits, and to organize ferritin molecules with no further decomposition.

Thus, the results of our experiments show that in peritoneal macrophages ferritin is synthesized with ferric hydroxide granules by taking up apoferritin molecules or their subunit proteins in the phagolysosomes, then the ferritin is dispersed diffusely into the cytoplasmic matrix. Whether peritoneal macrophages actually have a different pathway for ferritin synthesis from hepatocytes, or whether they use two different pathways for ferritin synthesis based on the chemical structure of the iron compounds taken into the cells is a matter for future study.

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