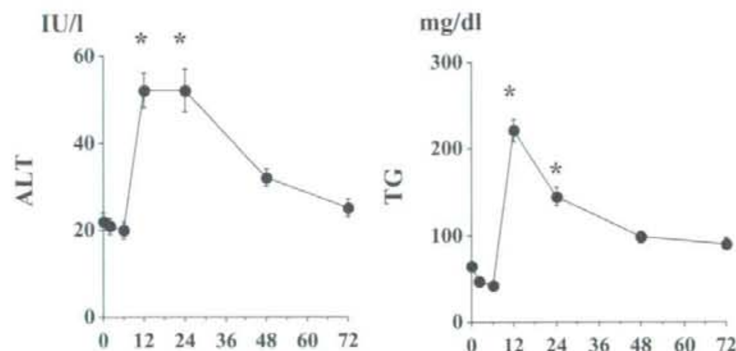

Fig. 2. Time-course change of biochemical markers alanine aminotransferase (*ALT*) and triglycerides (*TG*) in serum of mice administered 30 mg/kg of LPS and killed at several time points. Each data point represents the mean ± SEM of five animals. $*P < 0.01$, compared with control

sion was observed in mice injected with LPS at doses of 3 mg/kg or larger. We also examined the time-course effect of LPS on ADRP mRNA expression in the mouse liver (Fig. 5D). Real-time PCR demonstrated that ADRP mRNA expression in the liver increased by 6 h after a 30 mg/kg LPS injection. ADRP mRNA expression was gradually upregulated from 6 to 12 h after LPS administration, and then decreased to the baseline level, supporting our results on the time-course change of ADRP expression after LPS administration.

LPS also increased mRNA expression of TNFα in the liver within 2 h (Fig. 6).

PPARα and γ and RXRα are transcription factors that may be involved in accumulation of lipids in the liver.[8] PPARα, PPARγ, and RXRα mRNAs in the liver were drastically downregulated within 2 h after injection of 30 mg/kg LPS (Fig. 7). In contrast, SREBP-1 mRNA expression was not changed by LPS (Table 1).

To clarify whether fatty acid synthesis or lipid oxidation or both were involved in the accumulation of lipids in hepatocytes after LPS administration, we analyzed mRNA expression of several molecules that play a role in fatty acid synthesis or lipid oxidation in the liver (Fig. 8). LPS potently inhibited mRNA expression of the enzymes that play a key role in fatty acid synthesis, fatty acid synthase, stearoyl-CoA desaturase, and acetyl-CoA carboxylase α, suggesting that the lipid accumulation does not result from increased de novo synthesis of fatty acid. In contrast, mRNA expression of three enzymes involved in fatty acid oxidation, enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, was downregulated within 2 h after LPS administration.

Discussion

Although a number of studies have demonstrated that LPS affects biochemical markers of lipid metabolism in which the liver plays a key role,[1] it has not been known whether LPS induced distinct pathological changes in the liver. The present study demonstrated for the first time that intraperitoneal injection of LPS is capable of inducing transient lipid accumulation in the mouse liver. We therefore propose that LPS and inflammation should be listed as factors inducing liver steatosis.

As demonstrated in this study, LPS dose-dependently reduced body weight within 24 h. Although we did not examine the effect of LPS on food intake in this study, LPS-induced anorexia[9–11] is considered to be a factor in the body weight loss caused by LPS. One might speculate that reduced feeding by itself might be implicated in the lipid accumulation in the liver after LPS administration. However, that is unlikely because 24 h of fasting by itself failed to induce lipid accumulation in mouse liver in this study.

Histological observations indicated that lipid accumulation in the liver after LPS administration is stronger around the portal vein than around the central veins. We have recently demonstrated that a high fat diet causes lipid droplets to form in hepatocytes around the central veins in mice.[6] It is known that steatosis around the central veins is strong in fatty liver resulting from obesity or alcohol.[12] The observation that lipid accumulation occurred mainly in hepatocytes around the portal vein rather than the central veins as a result of LPS administration suggests that the mechanism of LPS-induced liver steatosis differs from that of well-established causative factors such as obesity or alcohol.

Lipid droplets are cytoplasmic organelles that serve as storage sites for neutral lipids. ADRP is intrinsically associated with the surfaces of lipid droplets and is believed to function in the intracellular mobilization and storage of neutral lipids.[13–15] ADRP abundance is directly proportional to the level of intracellular lipid found within a cell, and ADRP is increased in specific diseases involving fat accumulation.[13,16,17] Although there is little evidence for whether ADRP expression is indeed increased in fatty liver, in which the liver stores

Fig. 3. **A** Time-course change of liver tissues [hematoxylin and eosin (HE) staining] in mice injected with LPS. Mice were administered several doses of LPS intraperitoneally and then killed at several time points. **B** High-power view of liver tissue (HE staining) 24 h after LPS (30 mg/kg) injection. **C** Time-course change in liver tissue (oil red O staining) in mice injected with LPS. **D** High-power view of liver tissue (oil red O staining) 24 h after LPS (30 mg/kg) injection

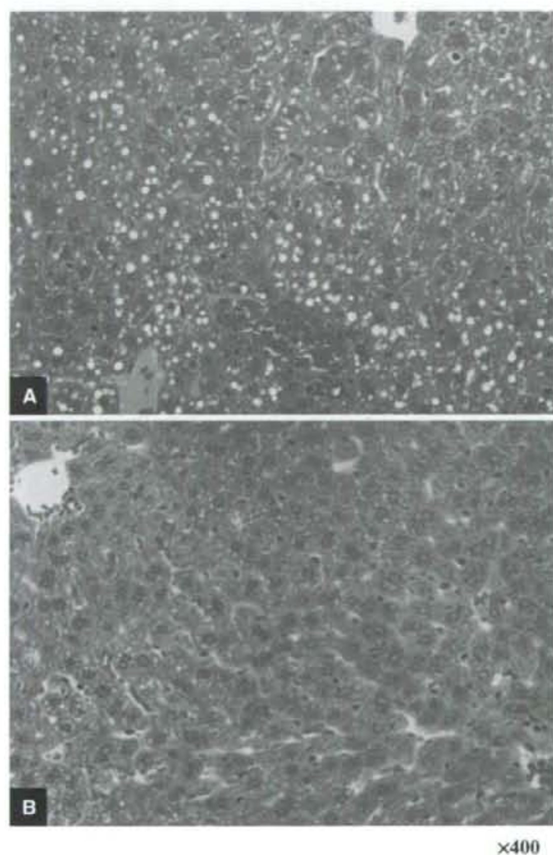

Fig. 4A,B. Effect of a 24-h fast on induction of lipid accumulation in the liver. Mice were deprived of food and then injected with LPS (30 mg/kg) (**A**) or not (**B**), and then killed after 24 h (HE staining)

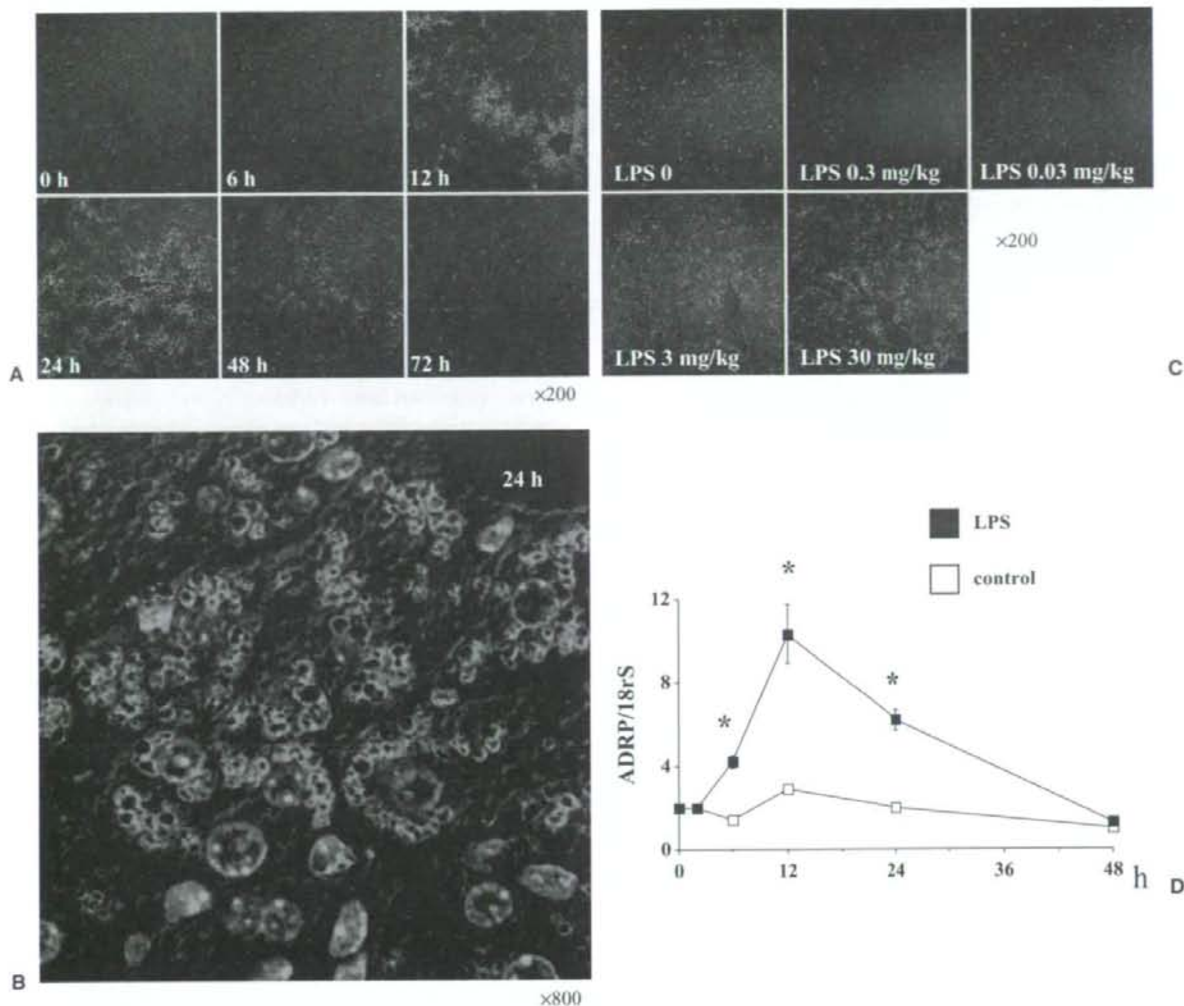

Fig. 5A–D. Representative adipose differentiation-related protein (*ADRP*) immunostaining of liver tissue in mice injected with LPS intraperitoneally. Mice were injected with several doses of LPS intraperitoneally and then killed at several time points. Liver tissues were stained with ADRP antibody. Time-course (**A**) and dose–response (**C**) effects of LPS on ADRP protein expression on the liver are shown. **B** High-power view of liver tissue (ADRP immunostaining) 24h after LPS (30mg/kg) injection. **D** Time-course effect of LPS on ADRP mRNA expression in the mouse liver. Mice were injected with a 30mg/kg dose of LPS intraperitoneally and then killed at several time points. ADRP mRNA expression in the liver was examined by real-time polymerase chain reaction (PCR). Each data point represents the mean ± SEM of five animals. *$P < 0.01$, compared with control

lipids, we recently demonstrated increased expression of ADRP in fatty liver in patients as well as in *ob/ob* and normal mice fed a high fat diet.[7] In addition, it has been very recently reported that an ADRP antisense oligonucleotide reduced liver steatosis in *ob/ob* and diet-induced obese mice,[18] and protection against fatty liver was observed in mice lacking ADRP,[19] strongly suggesting that ADRP plays a key role in the development of liver steatosis. The present study showed that LPS induced liver steatosis that was accompanied by increased expression of ADRP, indicating that upregulation of ADRP expression is a common molecular event during lipid accumulation in the liver, whatever its cause or mechanism.

Next, we investigated the mechanism by which LPS induces lipid accumulation in the liver in mice. Recent

studies have established a role for hepatic PPARγ in the development and maintenance of steatosis in the liver.[6,20–24] Upregulation of the PPARγ gene in the liver should result in steatosis, whereas lipid accumulation should be prevented by downregulation of the PPARγ gene in hepatocytes.[25–27] We therefore tested the hypothesis that PPARγ is involved in LPS-induced lipid accumulation in the liver. PPARγ mRNA expression in the liver was potently inhibited by LPS. Beigneux et al.[28] have shown that PPARγ mRNA in the liver is drastically reduced by LPS in hamster,[28] in agreement with the present data. These results led us to conclude that PPARγ is not implicated in the formation of lipid droplets in the liver by LPS.

PPARα also plays a key role in liver steatosis.[8,29] PPARα is abundantly expressed in the liver, where it functions as a lipid sensor and recognizes and responds to the influx of fatty acids by stimulating the transcription of PPARα-regulated genes.[30,31] These include genes encoding for mitochondrial, peroxisomal, and microsomal oxidation systems.[29] In conditions of increased demand for fatty acid oxidation, PPARα-null mice fail to upregulate fatty acid oxidation systems in liver with which to oxidize influxed fatty acids, and they develop liver steatosis.[32–34] Administration of PPARα agonists to rats not only prevents the development of methionine- and choline-deficient diet-induced steatohepatitis but also reverses steatohepatitis.[35,36] PPARα expression in the liver was rapidly and potently suppressed by LPS in

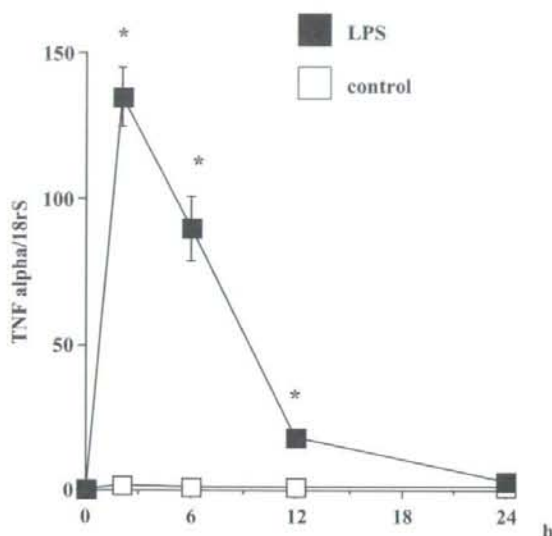

Fig. 6. Tumor necrosis factor (*TNF*) α mRNA expression in the liver was examined by real-time PCR 2, 6, or 12h after administration of 30mg/kg LPS to mice. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

Table 1. Time-course effect of LPS on SREBP-1 mRNA expression in the mouse liver

Time (h)	SREBP-1/18rS	
	control	LPS
2	1.75 ± 0.12	1.68 ± 0.08
6	2.12 ± 0.14	2.10 ± 0.11
12	2.01 ± 0.13	1.70 ± 0.15

Values are means ± SEM ($n = 5$)
LPS, lipopolysaccharide; SREBP-1, sterol regulatory element binding protein 1

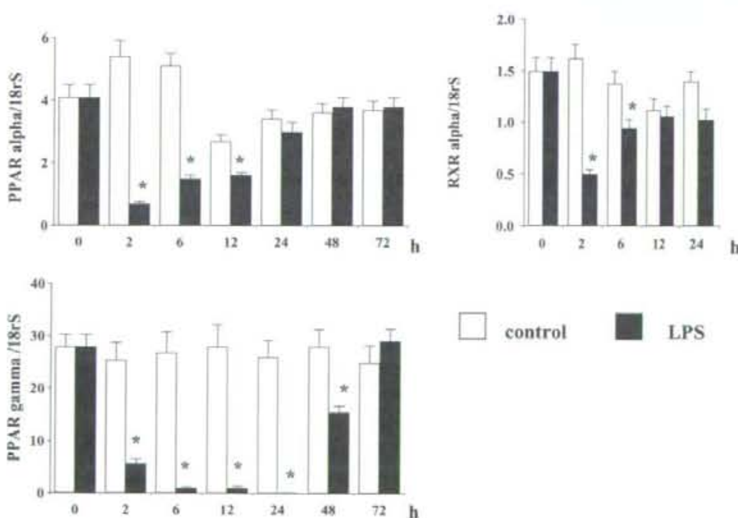

Fig. 7. Time-course change of mRNA expression of peroxisome proliferator-activated receptor (*PPAR*) α, PPARγ, and retinoid X receptor (*RXR*) α which are known to be transcription factors involved in lipid accumulation. Mice were injected with 30mg/kg LPS intraperitoneally and then killed at several time points. mRNA expression of PPARα, PPARγ, and RXRα in the liver was examined by real-time PCR. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

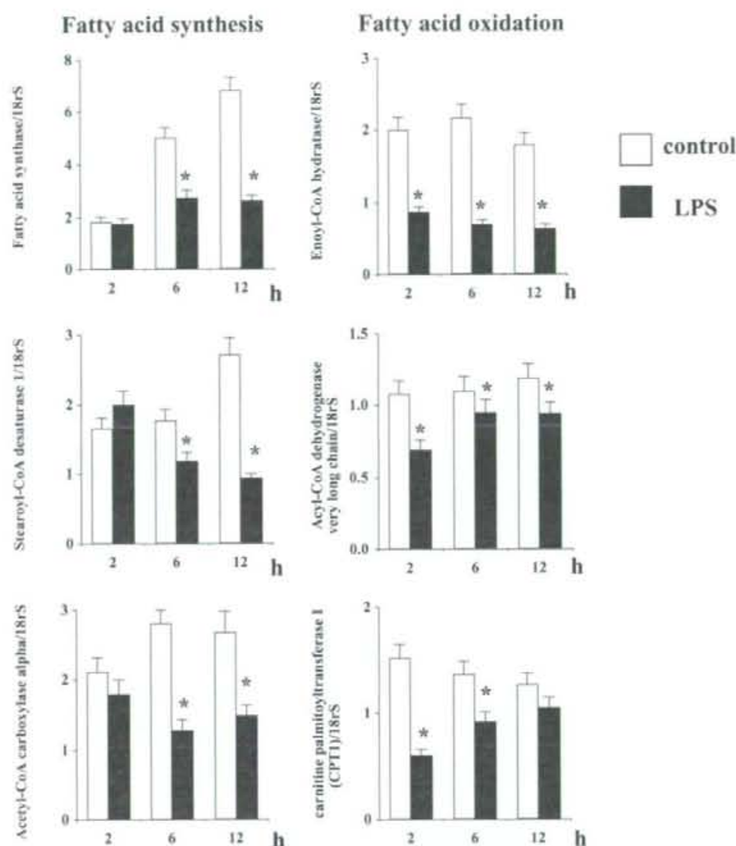

Fig. 8. Time-course change of mRNA expression of several enzymes that play a role in fatty acid synthesis or lipid oxidation in the liver: fatty acid synthase, stearoyl-CoA desaturase, acetyl-CoA carboxylase, enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1. Mice were injected with 30 mg/kg LPS intraperitoneally and then killed at several time points. mRNA expression of the above enzymes in the liver was examined by real-time PCR. Each data point represents the mean ± SEM of five animals. $^*P < 0.01$, compared with control

this study, as previously shown by Tai et al.[37] Considering this evidence, the present results suggest that reduced expression of PPARα in the liver may play a role in lipid accumulation caused by LPS.

LPS also inhibited the hepatic expression of RXRα, another nuclear hormone receptor. Because heterodimerization with RXRα is crucial for the action of PPARα,[38,39] the reduction of hepatic RXRα expression alone or in association with PPARα may be a mechanism for inhibiting the expression of multiple genes for lipid oxidation. The fact that decreased expression of transcription factors such as PPARα and RXRα occurred within 2 h after the LPS challenge supports the hypothesis that repression of those transcription factors plays a vital role in the accumulation of lipid in the hepatocytes caused by LPS. Because lipid accumulation was observed 12 h after LPS injection, LPS may suppress PPARα and its heterodimers, causing lipid accumulation in the liver through inhibition of PPARα-targeted genes for fatty acid oxidation, as described below.

Accumulation of lipids in the liver can result from either stimulation of lipid synthesis or inhibition of lipid oxidation. With regard to lipogenesis, de novo fatty acid synthesis in the liver is regulated by transcription factors such as SREBP-1c and PPARγ.[8] In this study, LPS inhibited expression of both these transcription factors in the liver, strongly suggesting that LPS-induced liver steatosis did not result from increased lipogenesis. In fact, our results showed that genes responsible for lipogenesis in the liver were not upregulated by LPS, further indicating that increased lipogenesis is not involved in LPS-induced liver steatosis.

Disruption of fatty acid oxidation can also account for excess lipid storage in the liver.[1] Some of the key enzymes of fatty acid oxidation systems in liver are regulated by PPARα.[29,31] In the present study, LPS potently inhibited expression of PPARα and its target genes, including enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, which are involved in fatty acid oxidation in the liver, indicating that reduced expression of PPARα plays a vital role

in LPS-induced lipid accumulation in the liver through inhibition of fatty acid oxidation.

Wolfe et al.[40] have demonstrated that sepsis and endotoxins increase hepatic very low density lipoprotein (VLDL) production. Several reports have demonstrated that an increase in hepatic VLDL production by sepsis or endotoxins may be caused by an increase in the reesterification of plasma fatty acids derived from the stimulation of lipolysis.[40-42] These findings suggest that an influx of fatty acid into hepatocytes might be involved in LPS-induced lipid accumulation. As clearly shown in the present study, LPS potently increased expression of TNFα mRNA in the liver. The increase in TNFα expression and reduced adipose tissue weight observed in this study after LPS administration suggest that lipolysis followed by an influx of fatty acids into hepatocytes might be involved at least in part in the observed hepatic fatty accumulation. Moreover, it has been reported previously that increased expression of TNFα induces lipolysis and hepatic lipid accumulation through downregulation of PPARα expression in the liver.[43,44]

Fatty acids undergo beta-oxidation in the mitochondria.[1] Some investigators have demonstrated abnormal mitochondrial function after endotoxic shock.[45-47] Therefore, we speculate that LPS may disrupt mitochondrial function, and that this disruption might play a role in the lipid accumulation in the liver, possibly through the inhibition of lipid oxidation. Further studies should be performed to address this possibility.

In summary, our results suggest that LPS induces transient lipid accumulation and expression of ADRP, a lipid droplet surface protein, in the liver through inhibition of fatty acid oxidation by downregulation of PPARα-related genes.

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Body iron metabolism and pathophysiology of iron overload

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Abstract Iron is an essential metal for the body, while excess iron accumulation causes organ dysfunction through the production of reactive oxygen species. There is a sophisticated balance of body iron metabolism of storage and transport, which is regulated by several factors including the newly identified peptide hepcidin. As there is no passive excretory mechanism of iron, iron is easily accumulated when exogenous iron is loaded by hereditary factors, repeated transfusions, and other diseased conditions. The free irons, non-transferrin-bound iron, and labile plasma iron in the circulation, and the labile iron pool within the cells, are responsible for iron toxicity. The characteristic features of advanced iron overload are failure of vital organs such as liver and heart in addition to endocrine dysfunctions. For the estimation of body iron, there are direct and indirect methods available. Serum ferritin is the most convenient and widely available modality, even though its specificity is sometimes problematic. Recently, new physical detection methods using magnetic resonance imaging and superconducting quantum interference devices have become available to estimate iron concentration in liver and myocardium. The widely used application of iron chelators with high compliance will resolve the problems of organ dysfunction by excess iron and improve patient outcomes.

Keywords Hemochromatosis · Hepcidin · Iron metabolism · Iron overload · Non-transferrin-bound iron (NTBI)

1 Introduction

Iron is an essential metal for hemoglobin synthesis of erythrocytes, oxidation–reduction reactions, and cellular proliferation, whereas excess iron accumulation causes organ dysfunction through the production of reactive oxygen species (ROS). The total amount of body iron is approximately 3–4 g, two-thirds of which is composed of red blood cell (RBC) iron and recycled iron by RBC destruction; the remainder is stored in ferritin/hemosiderin, while only 1–2 mg of iron are absorbed in the intestinal tract and circulated in the blood [1]. Body iron metabolism is a semi-closed system, and is critically regulated by several factors including the newly identified peptide hepcidin. In the circulation, iron is usually bound to transferrin (Tf), and most of the Tf-bound iron is utilized for bone marrow erythropoiesis [1]. As there is no active mechanism to excrete iron from the body, a progressive accumulation of body iron easily occurs as a result of long-term transfusions in patients with anemia of genetic disorders such as thalassemia, sickle cell disease (SCD), and Diamond Blackfan syndrome, and of bone-marrow failures such as aplastic anemia (AA) and myelodysplastic syndromes (MDS). In order to consider pathophysiological mechanisms of organ injury by iron overload, an understanding of molecular mechanisms of body iron metabolism is essential.

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Table 1 Molecules involved in body iron metabolism

Molecules
Molecules for intestinal iron absorption
Divalent metal transporter 1 (DMT1)
Duodenal cytochrome *b* (Dcytb)
Heme carrier protein (HCP)
Hemeoxygenase-1
Ferroportin
Hephaestin
Transferrin
Molecules for bone marrow iron uptake
Transferrin receptor 1
Transferrin
Molecules for reutilization of senescent red blood cells
Hemeoxygenase-1
Ferroportin
Transferrin
Molecules for hepatic iron storage
Ferritin
Hemosiderin
Transferrin
Transferrin receptor 1
Transferrin receptor 2
Non-transferrin-bound iron
HFE
β2-microglobin
Divalent metal transporter 1
ZIP14
Hemojuvelin
Molecules for systemic iron regulation
Hepcidin
(Unknown erythroid regulator?)

2 Molecular mechanisms of body iron metabolism

Table 1 shows a list of molecules involved in body iron metabolism, categorized as functions including intestinal absorption, erythroid iron uptake, reutilization of senescent RBCs, hepatic iron storage, and systemic regulation.

2.1 Intestinal iron absorption

Ingested iron is classified as non-heme iron and heme iron. Non-heme iron derived from plants is mainly composed of inorganic ferric Fe(III) iron, and is absorbed into enterocytes through the divalent metal transporter 1 (DMT1) after reduction of Fe(III) to Fe(II) by duodenal cytochrome *b* [2, 3]. In contrast, heme-iron derived from meat is absorbed through a heme carrier protein into enterocytes, where it is degraded by hemeoxygenase-1 (HO-1). Iron within enterocytes is then transferred from the luminal to the vascular site of the cell, and released into the circulation via the metal transporter, ferroportin in the form of Fe(II). Excreted Fe(II) is thereafter oxidized to Fe(III) by hephaestin, a homolog of ceruloplasmin, and the resulting ferric iron is bound to serum Tf [4].

2.2 Red blood cell iron reutilization in the reticuloendothelial system (RES) and iron load by blood transfusion

The average life span of circulating RBCs is approximately 120 days, indicating that 20 mg of iron derived from 20 ml of RBCs are processed by RES/macrophages on a daily basis. Within macrophages, heme derived from phagocytized RBCs is catabolized by HO-1, and free iron is released.

Intra-cellular iron is released into the circulation via ferroportin, and the iron is donated to Tf and reutilized for bone marrow erythropoiesis.

In patients with genetic anemias and bone marrow failures, regular transfusion is required in order to overcome the intractable symptoms. Transfused RBCs are taken up and degraded by RES/macrophages, in which the recycled iron is overloaded and the excess iron saturates the binding capacity of Tf. This excess iron appears in the circulation as a form of non-Tf-bound iron (NTBI) [1, 5], and causes organ dysfunction by the production of ROS. One milliliter of blood contains approximately 0.5 mg of iron, and there is no active mechanism for excretion of this excess iron. In Japan, one unit of blood corresponds to 200 ml of whole blood or 140 ml of concentrated RBCs, both of which contain approximately 100 mg of iron. As the critical level of iron overload at which organ dysfunction occurs in the liver is approximately 7 mg/g dry liver weight [6], according to the formula derived by Angelucci [body iron accumulation (mg/kg) = liver iron concentration (LIC; mg/g dry weight × 10.6)] [7], only 40 Japanese units of transfusion are required to reach this level.

2.3 Iron uptake and utilization in liver

The liver is a major storage organ of iron, in which excess iron is stored as ferritin and hemosiderin. In addition to these proteins, an additional fraction of free iron is present in the form of the labile iron pool (LIP) within cells. The LIP is biologically active in intracellular metabolism via oxidation–reduction reactions, cell proliferation, and cell signaling, but is toxic if present in excess. As shown in Fig. 1, hepatocytes have essentially two pathways for uptake of iron from the circulation: Tf-bound iron (Fe_2-Tf) at physiological iron concentrations, and NTBI in iron overload conditions [3].

Concerning the uptake of Fe_2-Tf, there are three pathways involved: two are dependent on and one is independent of transferrin receptor (TfR) recycling.

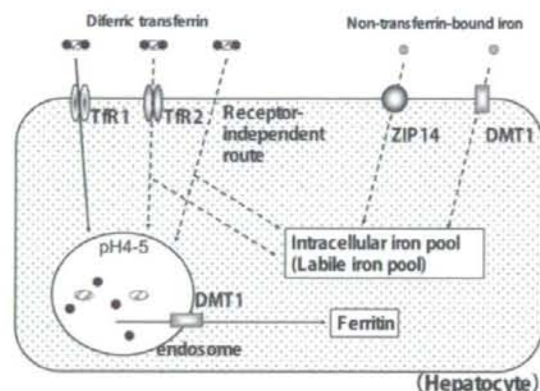

Fig. 1 Routes for iron uptake by hepatocytes. Hepatocytes have several pathways for iron uptake from the circulation. Concerning uptake of Tf-bound iron (Fe_2-Tf) at physiological concentrations, there are three pathways involving TfR1, TfR2, and TfR-independent mechanisms. The pathway via TfR1 is a classical one and is well elucidated. When serum Fe_2-Tf binds to TfR1, the Fe_2Tf-TfR1 complex is internalized by endocytosis, and iron is released within the endosome when endosomal pH is acidic. The resulting apotransferrin-TfR1 complex is then recycled back to the cell surface for re-utilization. Released iron into the endosome is transferred to the cytoplasm by DMT1; the resulting cytoplasmic free iron is used for iron-related biological functions, and the rest of the iron is stored as ferritin. In addition to TfR1, TfR2 and the mechanism that is independent of TfR1 and TfR2, are also considered to be important routes for iron uptake in hepatocytes, but the details of these routes remain to be elucidated. Concerning the hepatic uptake of NTBI, which is present in the serum during conditions of iron overload, DMT1 and ZIP14 are considered to be involved

Transferrin receptor 1 (TfR1) is a classical functional receptor, expressed highly in erythroblasts, but less so in hepatocytes. When serum Fe_2-Tf binds to TfR1, Fe_2-Tf is internalized by endocytosis. Internalized Fe_2Tf-TfR1 complexes within the endosome release iron when endosomal pH is acidified. The resulting apotransferrin-TfR1 complex is then recycled back to the cell surface for re-utilization. Transferrin receptor 2 (TfR2), a new homolog of TfR1, is ubiquitously expressed on hepatocyte surfaces and possesses a similar mechanism of recycling, but the binding affinity is rather weak: the functional role of TfR2 for cellular iron uptake is still obscured. In hepatocytes, there is another Fe_2-Tf uptake mechanism that is independent of TfR recycling, which is also considered to be important [8].

In iron-overloaded conditions, NTBI appears in the circulation and is taken up through two molecules such as DMT1 and ZIP14 on hepatocytes [9].

2.4 Bone marrow iron metabolism and erythropoiesis

Bone marrow erythroblasts require large amounts of iron for hemoglobin synthesis. TfR1 is strongly expressed in erythroblasts and functions as the uptake system of extracellular Fe_2-Tf. Within erythroblasts, iron is transferred to mitochondria and is incorporated into the center of the heme ring, which is synthesized by condensation of δ-aminolevulinic acid, a product made by erythroid δ-aminolevulinic acid synthase (eALAS). It is noteworthy that the synthesis of eALAS is also regulated by an iron-responsive-element binding protein (IRP) as well as TfR1 [10]. It is well known that genetic abnormalities of this pathway cause the phenotype of ringed sideroblastic anemias [11].

2.5 Systemic regulation of body iron metabolism

It has been postulated for a long time that a soluble factor acts to synchronize body iron metabolism between different organs. Recently, a basic peptide called hepcidin, an antimicrobial purified from urine, was found to have this role [12]. Hepcidin is considered to be a negative regulator that inhibits both intestinal iron absorption and reticulo-endothelial iron release. It is mainly synthesized in the liver, in which production is enhanced during iron overload and inflammation [13]. In some patients with genetic hemochromatosis, an abnormality of *hepcidin* gene has been reported. In these patients, hepcidin production was suppressed and iron absorption increased [14]. Furthermore, hepcidin expression is also down-regulated even in patients without a genetic abnormality of hepcidin. These reports strongly suggest that hepcidin plays an important role in tissue iron deposition in many iron-overloaded conditions including HFE hemochromatosis [15]. Currently, several additional molecules such as TfR2 and hemojuvelin (HJV) are also known to be involved in its regulation [16]. Furthermore, it is becoming clear that there is a role for hepcidin even in secondary iron overload. In a mouse model of β-thalassemia, representing ineffective erythropoiesis, there is an upregulation of hepcidin and a down-regulation of ferroportin, explaining how hepcidin also contributes to the formation of secondary hemochromatosis associated with ineffective erythropoiesis [17].

3 Forms of iron in serum and tissue

As free iron is extremely toxic to cells, the body has a number of protective mechanisms with which to bind iron in various tissue compartments. In serum, iron is usually bound to Tf, but some is present as NTBI when iron concentration exceeds the iron binding capacity of plasma Tf. It is also noted that ferritin is present in serum, although its biological role in iron transport is unclear.

3.1 Iron in plasma: Tf-bound iron and non-Tf-bound iron (NTBI)

It is well known that plasma Tf is capable of binding and transporting ferric iron to cells via TfRs. The binding capacity of Tf to inorganic iron is very strong, and this characteristic behavior prevents iron from existing in its free form under normal physiological conditions. As the Tf saturation in normal physiological conditions is up to 35%, this suggests that there is sufficient capacity to prevent the release of free toxic iron into the circulation [18]. However, when the iron binding capacity of Tf is saturated in the iron-overloaded state, an additional iron compartment NTBI, appears in the circulation. This compartment is biologically more toxic than Tf-bound iron. Among the NTBI fractions, labile plasma iron (LPI) is the most toxic. Unlike Tf-bound iron, the cellular uptake of NTBI is not dependent on the TfR, and therefore the resulting iron is diffusely distributed throughout the organs, independent of the presence of the TfR [5, 19]. Unlike serum iron, TIBC and percent-Tf-saturation measurements, the inter-institutional difference of NTBI and LPI measurements are too great and these parameters have not been standardized.

3.2 Iron in tissue: tissue ferritin and labile iron pool (LIP)

Within cells, iron is stored in the proteins ferritin or hemosiderin. Ferritin is a cytoplasmic protein consisting of 25 heterodimeric subunits of H and L that stores iron as ferric hydroxide phosphate in a controlled manner. Each molecule can store up to 4,500 Fe(III) within the protein shell [20], and release greater quantities of iron when the body is iron deficient. Most ferritin is present in liver, spleen, and bone marrow, and a trace amount is found in the blood as serum ferritin. It is noteworthy that the synthesis of ferritin is post-transcriptionally regulated by the cytoplasmic transacting factor IRP. IRP activates ferritin synthesis when iron is excess in the cell [21]. This adaptive response is important for preventing cells from free iron toxicity.

In addition to ferritin iron, LIP is present within cells in order to facilitate biological actions involving iron atoms, and can become cytotoxic or carcinogenic when the concentration exceeds the protective capacity of ferritin. Most of the LIP is free ferric iron bound to citrate or adenosine diphosphate, and a small amount of LIP is reduced to ferrous iron, which is responsible for oxidation–reduction reactions and the Fenton reaction. Iron toxicity is developed thorough the production of ROS.

3.3 Serum ferritin

In 1972, Jacobs et al. [22, 23] in the UK reported that ferritin was also present in serum, although its amount was very low. By quantitative phlebotomy, it was found that serum ferritin (SF) correlated with total body iron stores. Although it is still not clear how SF is produced, it is the most convenient laboratory test available to estimate body iron stores at the present time. However, the level of SF is also affected by acute and chronic inflammation and infections. Therefore, data should be interpreted carefully when using SF as a biological marker for evaluation of body iron stores, as shown in Table 2. There is a difference between the standard values of SF concentration in males and females (normal range 10–220 μg/L in males; 10–85 μg/L in females). It is clear that low SF values less than

Table 2 Considerations needed to use serum ferritin as a biological marker for the evaluation of body iron store

- **There is a sex difference of standard values of serum ferritin concentration**
  - ✓ Male: 10–220 μg/L, Female: 10–85 μg/L
- **Serum ferritin will be increased in various clinical conditions other than iron overload**
  - ✓ Chronic inflammation (effect of inflammatory cytokines)
  - ✓ Chronic liver damage (release from destroyed hepatocytes)
  - ✓ Malignancies (release from destroyed tumors)
  - ✓ The conditions needed to be considered for differential diagnosis dependent on the value of serum ferritin
    - **Slight elevation (250–500 μg/L)**
      - Malignancies, chronic liver damage, chronic inflammation, mild iron overload
    - **Mild elevation (500–1000 μg/L)**
      - Early stage of iron overload, ineffective erythropoiesis (thalassemia, etc)
      - The frequency of the conditions except iron overload decreases
    - **Moderate elevation (1000–5000 μg/L)**
      - Iron overload, Adult Still's disease, hemophagocytic syndrome
    - **Severe elevation (more than 5000 μg/L)**
      - Iron overload (hemochromatosis)

12 μg/L are usually representative of body iron deficiency. On other hand, patients with SF levels that are higher than the normal range may be indicative of conditions such as iron overload, inflammation, collagen disease, malignancy, and hepatic diseases [24]. This characteristic feature of the SF assay is considered to be a disadvantage for monitoring iron overload. Especially in Japan, the significance of SF as an inflammation marker has been over-stressed because there are few patients with hereditary hemochromatosis showing significantly high values of more than a couple of thousand or ten thousand microgram per liter.

Systemic measurements of SF in various diseases were conducted mainly in the late 1970s, just after the development of this assay, and it was found that AA and sideroblastic anemia patients who had received blood transfusions had SF levels of more than 1,000 μg/L, whereas patients without transfusions had lower levels. These old data have suggested previously that anemic patients who had ineffective erythropoiesis without transfusion support could maintain their SF levels at values less than 1,000 μg/L, even though adaptive increases in intestinal iron absorption were noted [25]. Therefore, the interpretation of the value of SF for the assessment of body iron status is simplified if other clinical conditions such as inflammation and malignancy are excluded by other modalities. The clinical studies concerning the relationship between blood transfusion and SF have been conducted mainly in the Europe and US, showing that there is a clear-cut positive correlation between the amount of chronic blood transfusion and the elevation of SF in patients with β-thalassemia [26, 27]. Furthermore, the concentration of heart iron is increased when SF levels become greater than 1,800 μg/L, and the prevalence of cardiac events is significantly increased when SF levels are more than 2,500 μg/L [6, 28]. Similar results concerning the relationship between SF and organ dysfunction of liver and heart were shown in a Japanese retrospective study in transfusion-dependent patients with bone-marrow-failure syndromes [29]. In this study, 90% of patients with either cardiac or hepatic complications had high SF levels of more than 1,000 μg/L. Coincidentally, this level of SF also represents the threshold of the target value at which iron chelation therapy should be initiated in patients with transfusion iron overload, according to the guidelines of the International MDS Symposium [30].

4 Measurement of body iron stores: comparison with serum ferritin

Direct and indirect methods are available for the estimation of body iron. As previously mentioned, the measurement of SF is the most convenient and cost-effective technique, although other factors can also influence its value. There is no argument that the gold standard for iron determination is direct tissue iron determination. Notably, other methods that are becoming increasingly important include physical methods such as the superconducting quantum-interference device (SQUID) and magnetic resonance imaging (MRI).

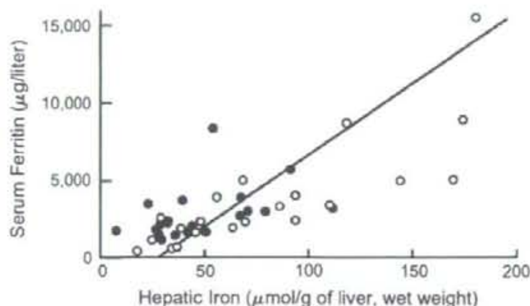

Fig. 2 Comparison of hepatic iron and serum ferritin concentrations. Indirect estimation is compared with the reference method, based on the direct measurement of hepatic iron levels by chemical analysis or magnetic-susceptibility studies. *Open circles* denote the values at the start of the trial (before deferiprone therapy), and *solid circles* the values at the time of the final analysis. The *diagonal line* denotes the simple linear least-squares regression between the two variables. (From [31]. Reproduced with permission. Olivieri NF et al. N Engl J Med. 1995;332:918–22. Copyright ©1995 Massachusetts Medical Society. All rights reserved)

4.1 Direct measurement

Liver is the major organ for iron storage and has the largest capacity to store excess iron. The measurement of hepatic iron concentration by liver biopsy is the most reliable means to assess body iron storage; however, this procedure is invasive and cannot be used in all cases [7]. Figure 2 compares the indirect estimation of body iron based on serum ferritin and LIC. Open circles denote the values at the start of the trial (before treatment with deferiprone), and solid circles denote the values at the time of the final analysis. The correlation between these measurements was significant ($R = 0.73$; $P < 0.005$) [31]. Concerning the determination of cardiac iron deposition, myocardial biopsy can be used; however, this procedure is not often conducted without special experimental reasons due to its high technical risk.

In patients with β-thalassemia, there is a correlation between LIC and cumulative amounts of RBC transfusions [26] and the risk of organ dysfunction is enhanced when LIC values are greater than 7 mg/kg wet tissue, and LIC levels of over 15 mg/kg wet tissue increase the risk of early cardiac death due to iron deposition in the myocardium [6]. Studies in the deferasirox clinical development program in β-thalassemia also demonstrated a correlation between the reduction in LIC and SF values ($R = 0.63$).

4.2 Physical measurement of body iron

As iron is one of the heavy metals, an increased concentration of biological iron consisting of ferritin and hemosiderin can be detected by body imaging procedures. Until recently, abdominal echograms and computed tomography (CT) produced images at high iron concentrations, although these two modalities are not quantitative and are only capable of detecting iron overload under conditions of extremely high iron deposition [32]. Recently, quantitative procedures such as SQUID [33] and MRI have been introduced, which use the physical characteristics of iron. However, SQUID apparatus is only available in a couple of institutions in the Europe and US because of its cost. On other hand, LIC determinations by MRI are widely available. This method utilizes the specific characteristic of iron that shortens T1, T2, and T2* relaxation times. The measurable range of iron concentration by R2 (in a 1.5-T MRI magnet) is 0.3–42.7 mg Fe/g dry tissue, which covers the concentrations observed in iron-overloaded livers.

In addition to LIC measurement, the determination of cardiac iron concentration is clinically important because one of the major causes of death in iron overload is sudden cardiac arrest. The most reliable non-invasive method of cardiac iron is MRI R2*, which was developed by Anderson et al. [34]. The advantage of MRI R2* is the shorter time period required to acquire an image as only one breath period is necessary by this procedure.

Of the patients with LIC values below 350 μmol/g, all but one had myocardial iron within normal ($\leq$8 μmol/g) or nearly normal ranges. When liver iron levels reached a threshold of 350 μmol/g, iron deposition became evident in the myocardium. At the same time, there was a proportional increase in urinary iron excretion, indicating raised levels of labile iron. SF levels of $>$1,800 μg/L were also associated with myocardial deposition.

5 Toxic effect of iron overload on organ function

Iron overload induces organ damage in liver, heart, pancreas, thyroid, and the central nervous system. The main cause of this organ damage is due to the overproduction of ROS in the presence of excess iron.

5.1 Mechanism of iron toxicity

The production of ROS by iron is mainly through the Fenton reaction, which eventually forms hydroxyl radicals from superoxide or hydrogen peroxide [35]. Among ROS, the hydroxyl radical is the most toxic fraction and it targets carbohydrate, protein, and nucleic acids. It is known that the reaction of hydroxyl radicals with the nucleic acid base 8-hydroxyguanine (8-OHG) is highly correlated with teratogenicity and carcinogenicity by oxidative stresses. Another powerful ROS showing similar reactivity as the hydroxyl radical is lipid hydroxyl-peroxide: ROOH. In iron overload, lipid peroxidative products such as malondialdehyde and 4-hydroxy-2-nonenal are increased, which form the radicals ROO-(alkyl oxyradical) and RO-(alkoxy radical). These lipid-based radicals possess longer half lives than hydroxyl radicals, and also have a stronger capacity for chronic cell toxicity and DNA damage.

5.2 Iron overload syndrome

Pathological conditions representing body iron overload are designated as iron overload syndromes, and iron deposition causes organ dysfunction including cell death, fibrosis, and carcinogenesis. Iron overload syndromes are classified as genetic or secondary as shown in Table 3.

Hereditary hemochromatosis is the most common genetic disorder in Western countries [36], and its clinical

Table 3 Classification of iron overload

Hereditary hemochromatosis and related disorders	
Hereditary hemochromatosis	Type 1
	HFE gene (6p21.3) mutation
	Type 2
	Subtype A: *hemojuvelin* gene (1q21) mutation
	Subtype B: *hepcidin* gene (19q13) mutation
	Type 3
	Transferrin receptor 2 gene (7q22) mutation
	Type 4
	Ferroportin gene (*2q32*) mutation
Ferritin gene mutation	*H-ferritin* gene mutation (mRNA iron-responsive-element mutation)
DMT1 gene mutation	
Ceruloplasmin gene mutation	
Atransferrinemia	*Transferrin* gene mutation
Secondary iron overload	
Ineffective erythropoiesis	Thalassemia, sideroblastic anemia, myelodysplastic syndromes
Administration of iron for long periods	Take orally or intravenous injection
Transfusion for long periods	
Dietary iron overload	
Liver dysfunction	Alcoholic liver injury, chronic hepatitis (type C), non-alcoholic steatohepatitis
Others	Porphyria

manifestation is systemic iron deposition mainly in liver, heart, brain, and endocrine organs. This organ damage is considered to be a result of tissue injuries by iron-induced oxidative stresses [37]. In 1996, the causative gene was identified as *HFE* in the human chromosome 6 [38], and approximately 85% of patients with hereditary hemochromatosis in Western counties have a homologous mutation of C282Y in their *HFE* gene. Thereafter, other genes such as *hemojuvelin* (*HJV*), *TfR2*, *ferroportin*, and *hepcidin* (*HAMP*) gene were identified [39]. In spite of the lack of genetic background, iron overload is commonly observed as a secondary condition. The most common condition occurs in patients who require long-term blood transfusions due to severe anemias. This condition includes genetic disorders such as thalassemia and SCD, and anemia refractory to conventional treatments. In these patients, ineffective erythropoiesis and continuous accumulation of exogenous iron by transfusion are considered to be responsible for the iron overload. The resulting organ failures such as liver failure, cardiac failure, and severe diabetes mellitus affect patients' outcome [1]. In addition to these classical conditions, there are many diseases that show mild iron deposition or dysregulation of body iron distribution. Such conditions include chronic hepatitis C, alcoholic liver disease, non-alcoholic steatohepatitis, and insulin resistance, and iron is an important cofactor that modifies these disease conditions. Furthermore, it is becoming clear that excess iron is also hazardous as it promotes atherosclerosis, carcinogenesis, diabetes, and other lifestyle-related disorders [40].

5.3 Organ dysfunction by excess iron

The liver is the most important organ for iron storage with the largest capacity to sequester excess iron. The periodical change of organ dysfunction by long-term transfusions has been studied in patients with homozygous β-thalassemia. Usually, within 2 years of transfusion, abnormalities of liver function tests (LFTs) such as transaminase are not prominent; LFTs are within the normal range or slightly elevated. During these periods, the liver biopsy examination shows a slight fibrosis with mild inflammation and iron deposition. Clinically, the liver is hardened and palpable, and serum transaminase levels are moderately elevated, while other LFTs are within the normal range or slightly elevated. Therefore, it is important for transfusion-dependent patients that clinicians make a correct staging in order to confirm whether any liver lesions are fibrotic or cirrhotic by examining CT, MRI, and biochemical analyses including serum transaminase determinations.

The most important adverse event of long-term transfusion is a sudden death due to cardiac failure. It was reported that approximately 70% of deaths in patients with β-thalassemia are cardiogenic [41]. Signs of cardiac dysfunction include cardiac hypertrophy, arrhythmia, and endocarditis, which eventually cause cardiac failure. Left ventricular disturbance is prominent and is represented as the decrease of ventricular ejection fraction (VEF) by cardiac echogram. As this decrease of VEF appears prior to the clinical signs of cardiac failure and the enlargement of cardiac shadow in chest X-rays, the cardiac echogram is the most useful modality for the follow-up of myocardial damage by iron overload [42]. MRI is also useful to assess the ventricular function, and the deposition of iron in cardiac muscles is detectable by an increase in signal intensity. Furthermore, MRI calculation of T2* or R2* allows the possibility of semi-quantitation of iron concentrations, even at relatively low concentrations [43].

According to a follow-up study in patients with β-thalassemia, organ dysfunction by iron overload appears firstly in the liver when serum ferritin exceeds 1,000 μg/L, and other organ involvements including heart follow in accordance with the further development of iron deposition. Significant cardiac iron deposition is usually observed when LICs are more than 15 mg/g dry weight or serum ferritin levels are more than 1,800–2,500 μg/L [6].

Clinically, in order to detect organ dysfunctions, serum ferritin determinations should be conducted once every 1–3 months. When serum ferritin levels exceed 1,500 μg/L, patients should be examined for the symptoms of cardiac failure or arrhythmias [44], and periodical cardiac echograms may also be useful in diagnosis.

In addition to iron deposition in the liver and heart, pancreatic beta cells are another important target of iron toxicity, which cause glucose intolerance and diabetes mellitus. An additional factor leading to the development of glucose intolerance is hepatic disturbance of insulin utilization, which accelerates beta cell depletion due to hyperinsulinemia [45]. From a clinical perspective, serial determinations of blood glucose, urine sugar, and glycoalbumin are useful, whereas glycohemoglobin is not as useful owing to the effect of transfusions. Endocrinopathies by long-term transfusion include developmental disturbances, incomplete puberty, and thyroid dysfunctions [46]. In patients with thalassemia and SCD, special attention should be paid to early onset symptoms such as disturbances of development and sexual immaturity.

6 Conclusion

Iron is essential for the body, but extremely toxic when excess amounts are present. As the body has no active excretion pathways for iron, a continuous load of iron exceeding 1–2 mg/day will result in iron overload, and organ failures including liver and heart. The recent

understanding of body iron metabolism at a molecular level enables us to elucidate the mechanism of iron toxicity more precisely. Improvement of patients' outcomes is becoming promising if a correct early diagnosis is made, and suitable management of these intractable conditions using iron chelation with high compliance is conducted.

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Association between metabolic syndrome and carotid atherosclerosis in individuals without diabetes based on the oral glucose tolerance test

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ABSTRACT

Introduction: Whether or not metabolic syndrome is predictive of atherosclerotic disorders may depend on the population studied. We investigated whether metabolic syndrome is associated with carotid atherosclerosis in individuals who were shown not to have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).

Methods and results: Between 1994 and 2003, 3904 individuals underwent general health screening that included the OGTT. Among these 3904 individuals, 3679 had a fasting plasma glucose of <126 mg/dL (subgroup 1), and 3488 had a 2-h post-OGTT glucose value of <200 mg/dL (subgroup 2). In both subgroups, metabolic syndrome was found to be a risk factor for carotid plaque and for carotid intima-media thickening in men, and tended to be a risk factor for carotid plaque in women after adjustment for age. Among 3473 individuals who had both a fasting plasma glucose value of <126 mg/dL and a 2-h post-OGTT glucose of <200 mg/dL, 2440 did not have hypertension, which was defined as systolic and diastolic blood pressure of <140/90 mmHg and absence of use of anti-hypertensive medication. In these non-diabetic non-hypertensive individuals, the association between metabolic syndrome and carotid plaque or carotid intima-media thickening was not statistically significant even with adjustment only for age.

Conclusions: In men who did not have impaired fasting glycemia and/or in those without impaired glucose tolerance, metabolic syndrome was a predictor of carotid atherosclerosis after age adjustment, although metabolic syndrome was not found to be a predictor of carotid atherosclerosis when hypertensive individuals were excluded from the study population.

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1. Introduction

Metabolic syndrome (MetS) is a cluster of metabolic and hemodynamic abnormalities linked with insulin resistance. Since components of MetS also represent risk factors for atherosclerotic disorders, it is natural that individuals with this syndrome have an increased risk for ischemic heart disease [1] and stroke [2,3]. On the other hand, the clinical utility of MetS may depend on whether the risk conveyed by this syndrome is higher than the sum of each component utilized as diagnostic criteria for MetS [4,5].

Carotid artery intima-media thickness has been reported to be a discriminator as a surrogate of cardiovascular mortality in community-dwelling Japanese people [6] and, conversely, aggregation of established major coronary risk factors has been reported to strongly influence the presence of carotid atherogenesis in the general Japanese population [7]. Previously, we reported that the presence of MetS may not increase the risk for carotid atherosclerosis in individuals without hypertension, with hypertension defined as systolic blood pressure (SBP) of ≥140 mmHg, diastolic blood pressure (DBP) of ≥90 mmHg, or the use of anti-hypertensive medication [8]. This observation suggested that the properties of MetS that present a risk for atherosclerotic diseases may differ according to the populations selected. Consistent with this idea, it was reported that MetS was not found to be associated with cardiovascular mortality in non-diabetic non-hypertensive Chinese individuals [9], and that MetS did not significantly increase the risk of mortality from cardiovascular disease in non-diabetic Mexican Americans and non-Hispanic whites [10]. In the current study, we investigated whether MetS was associated with carotid atherosclerosis in Japanese individuals who did not have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).

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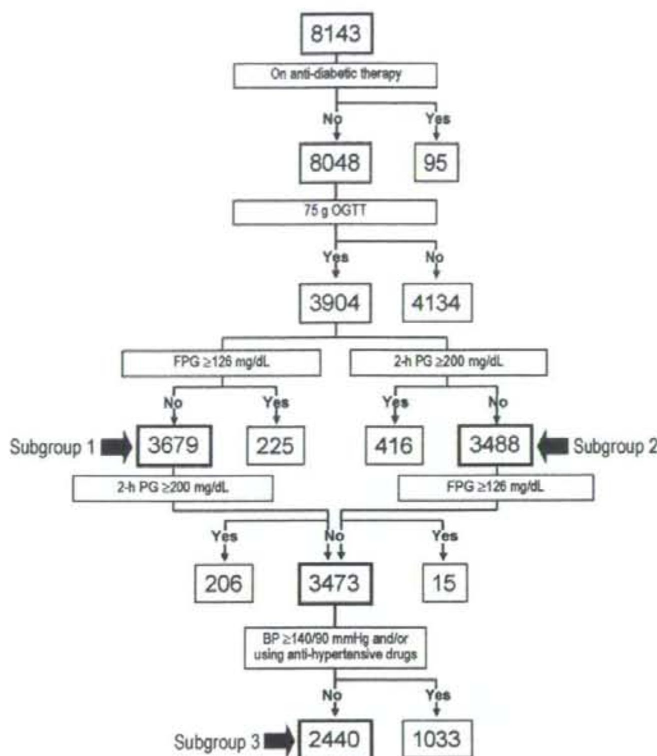

Fig. 1. Flow chart showing selection of the four subgroups.

2. Methods

2.1. Study subjects and selection of subgroups

The study was approved by The Ethical Committee of Mitsui Memorial Hospital and University of Tokyo, Faculty of Medicine. Between September 1994 and December 2003, 8143 subjects underwent general health screening including carotid ultrasonography at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. Of the 8143 subjects, 95 were treated as having diabetes, and of the remaining 8048 individuals, 3904 underwent an OGTT. Among these 3904 individuals, three subgroups were sequentially selected based on various parameters (Fig. 1). Those with a fasting plasma glucose (FPG) value of <126 mg/dL were designated as subgroup 1, and those with a 2-h post-OGTT plasma glucose (2-h PG) value of <200 mg/dL were designated as subgroup 2. Subgroup 3 was comprised of subjects who met all the following conditions: FPG of <126 mg/dL, 2-h PG of <200 mg/dL, and not having hypertension. Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or the use of anti-hypertensive medication. We also selected individuals without impaired glucose tolerance (IGT), i.e., individuals with a 2-h PG value of <140 mg/dL.

At our institute, several types of health screening programs are available, and some general health screening programs include carotid ultrasonography and/or OGTT, while others do not. However, the decision on the type of health screening was made by the individuals and/or their companies and was not decided upon or recommended by any attending physician.

2.2. Definition of MetS

MetS was defined as the presence of three or more of the following: (1) fasting glucose ≥110 mg/dL; (2) SBP/DBP ≥130/85 mmHg or taking anti-hypertensive medication; (3) triglycerides ≥150 mg/dL mmol/L; (4) HDL cholesterol <40 mg/dL in men and <50 mg/dL in women; and (5) body mass index ≥25 kg/m^2 [11].

2.3. Carotid ultrasonography

Carotid artery status was studied using high resolution B-mode ultrasonography (Sonolayer SSA270A, Toshiba, Japan) equipped with a 7.5 MHz transducer as described previously [12]. Plaque was defined to be present when there is one or more clearly isolated focal thickening(s) of the intima-media layer with thickness of ≥1.3 mm at the common or internal carotid artery or the carotid bulb. Carotid wall intima-media thickening was said to be present when intima-media thickness which was measured at the far wall of the distal 10 mm of the common carotid artery was ≥1.0 mm [12].

2.4. Statistical analysis

Logistic regression analysis was used to obtain adjusted odds ratios and their 95% confidence intervals (CIs) to predict the presence of carotid plaque or carotid intima-media thickening. Statistical analyses were carried out by using Dr. SPSS II (SPSS Inc., Chicago, IL). Results are expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was taken to be statistically significant.

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Table 1
Baseline characteristics.

Variables	Subgroup 1		Subgroup 2		Subgroup 3	
	Men	Women	Men	Women	Men	Women
Number	2548	1131	2386	1102	1588	852
Age, years	58.2 ± 10.6	57.9 ± 10.4	58.0 ± 10.7	57.8 ± 10.3	56.7 ± 10.9	56.6 ± 10.5
Body mass index, kg/m^2	24.0 ± 2.8	22.2 ± 3.1	23.9 ± 2.7	22.1 ± 3.1	23.6 ± 2.6	21.7 ± 2.8
Systolic BP, mmHg	127 ± 19	121 ± 21	128 ± 19	120 ± 20	119 ± 12	123 ± 14
Diastolic BP, mmHg	79 ± 12	73 ± 12	79 ± 12	73 ± 12	73 ± 8	69 ± 9
Total cholesterol, mg/dL	206 ± 32	219 ± 35	205 ± 32	219 ± 35	205 ± 32	216 ± 35
HDL-cholesterol, mg/dL	55 ± 16	70 ± 17	55 ± 16	70 ± 17	56 ± 16	71 ± 17
Triglycerides, mg/dL	144 ± 117	96 ± 56	142 ± 98	95 ± 54	141 ± 98	95 ± 54
Uric acid, mg/dL	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.6 ± 1.0
Fasting glucose, mg/dL	96 ± 10	90 ± 10	95 ± 10	90 ± 9	94 ± 9	88 ± 9
2-h OGTT glucose, mg/dL	132 ± 41	118 ± 32	125 ± 29	115 ± 26	121 ± 29	112 ± 25
Haemoglobin A1C, %	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4
Hypertension, n (%)	863 (34)	263 (23)	788 (33)	248 (23)	0	0
Anti-hypertensive drugs, n (%)	336(13)	99(9)	307(13)	95(9)	0	0
Metabolic syndrome, n (%)	439(17)	84(7)	372(16)	72(7)	131 (8)	25(3)
Smoking status						
Never, n (%)	764 (30)	933 (82)	714(30)	909 (82)	465 (29)	689(81)
Former, n (%)	799(31)	53(5)	753 (32)	50(5)	464 (29)	44(5)
Current, n (%)	985 (39)	145(13)	919(39)	143(13)	659(41)	119(14)

BP indicates blood pressure, OGTT indicates oral glucose tolerance test.

3. Results

3.1. Association between MetS and carotid atherosclerosis in individuals with FPG value of <126 mg/dL (subgroup 1)

Among the 3904 individuals who underwent OGTT, 3679 (94%) had an FPG value of less than 126 mg/dL. Of these, 300 (257 men, 43 women), the FPG value was ≥110 mg/dL, thus impaired fasting glycemia (IFG), and in the remaining 3379 (2291 men, 1088 women) had an FPG value of less than 110 mg/dL (no IFG). Table 1 shows the baseline characteristics of this group according to gender. Carotid plaque was found in 823 (32%) men and 191 (17%) women and carotid intima-media thickening was found in 422 (17%) men and 122 (11%) women (Fig. 2). Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 1) and intima-media thickening (Table 2). In women, MetS tended to be associated with carotid plaque, but not with intima-media thickening after age adjustment. Similar patterns of relationships could be observed after further adjustment for total cholesterol (TC) and smoking status (Model 3). On the other hand, after full adjustment including that for components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in either men or women.

3.2. Association between metabolic syndrome and carotid atherosclerosis in individuals with 2-h PG value of <200 mg/dL (subgroup 2)

Among 3904 individuals who underwent OGTT, 3488 (89%) had a 2-h PG value of less than 200 mg/dL. Of these 3488 individuals 2644 (1717 men, 927 women) had a 2-h PG value of less than 140 mg/dL (no IGT) and the remaining 844 (669 men, 175 women) had a 2-h PG FPG value of ≥140 mg/dL, and thus IGT. Carotid plaque was found in 761 (32%) men and 182 (17%) women and carotid intima-media thickening was found in 378 (16%) men and 116 (11%) women. Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 2) and intima-media thickening (Table 3). In women, MetS tended to be associated with carotid plaque but not with intima-media thickening. Similar patterns of relationship could be observed after further adjustment for TC and smoking status (Model 3). On the other hand, after full adjustment that included components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in men or in women. There were only 15 (13 men, 2 women)

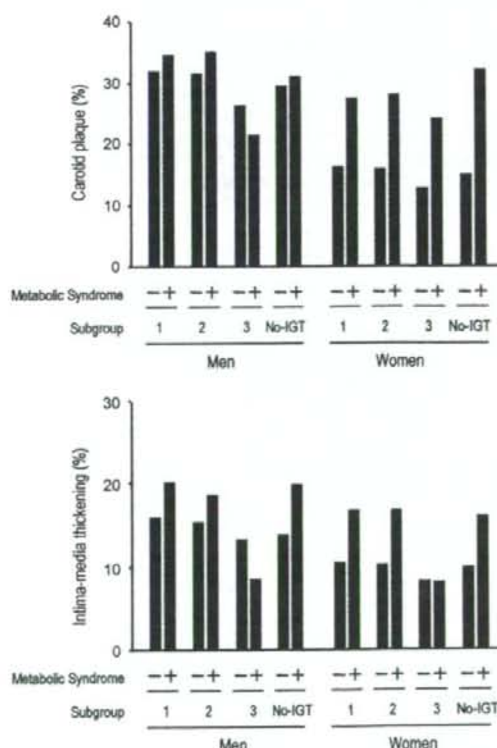

Fig. 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in subgroups.

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Table 2
Logistic regression analysis with metabolic syndrome as an independent variable and carotid plaque as a dependent variable.

Variables	Odds ratio for carotid plaque			
	Men		Women	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Subgroup 1				
Model 1	1.12(0.90–1.39)	0.302	1.97(1.19–3.28)	0.009
Model 2	1.41(1.11–1.79)	0.005	1.68(0.96–2.95)	0.072
Model 3	1.30(1.03–1.67)	0.030	1.63(0.93–2.88)	0.091
Model 4	1.21(0.90–1.63)	0.209	1.61(0.79–3.29)	0.188
Subgroup 2				
Model 1	1.18(0.93–1.49)	0.170	2.06(1.20–3.55)	0.009
Model 2	1.47(1.14–1.90)	0.003	1.78(0.98–3.24)	0.058
Model 3	1.38(1.07–1.78)	0.014	1.72(0.95–3.14)	0.076
Model 4	1.23(0.90–1.69)	0.202	1.73(0.82–3.63)	0.151
Subgroup 3				
Model 1	0.77(0.50–1.19)	0.232	2.20(0.86–5.62)	0.101
Model 2	0.99(0.62–1.58)	0.971	1.89(0.66–5.43)	0.235
Model 3	0.94(0.59–1.50)	0.796	1.85(0.64–5.33)	0.254
Model 4	0.82(0.48–1.41)	0.479	2.44(0.72–8.29)	0.152

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

individuals among the 3488 in subgroup 2 who had an FPG value of <126 mg/dL in addition to a 2-h PG value of <200 mg/dL, and, thus, the mode of association between MetS, carotid plaque, and intima-media thickening in this subgroup was essentially the same as that observed in total population of subgroup 2.

We also investigated the association between MetS and carotid atherosclerosis in individuals without IGT. There were 2644 individuals who did not have IGT, and among them, 61 had FPG value of ≥110 mg/dL (Fig. 2, Supplementary Tables 1 and 2). The obtained results in these subgroups were similar to those in the subgroup 2; however, association between MetS and carotid intima-media thickening was statistically significant even after multivariate adjustment in women.

Table 3
Logistic regression analysis with metabolic syndrome as an independent variable and carotid intima-media thickening as a dependent variable.

Variables	Odds ratio for carotid intima-media thickening			
	Men		Women	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Subgroup 1				
Model 1	1.33(1.03–1.73)	0.031	1.74(0.95–3.19)	0.074
Model 2	1.74(1.31–2.30)	<0.001	1.40(0.72–2.73)	0.324
Model 3	1.65(1.24–2.19)	<0.001	1.38(0.70–2.70)	0.349
Model 4	0.97(0.67–1.39)	0.851	0.70(0.31–1.60)	0.398
Subgroup 2				
Model 1	1.26(0.94–1.68)	0.120	1.78(0.93–3.42)	0.083
Model 2	1.63(1.20–2.22)	0.002	1.47(0.73–2.98)	0.285
Model 3	1.55(1.13–2.11)	0.006	1.44(0.71–2.93)	0.317
Model 4	1.00(0.68–1.48)	0.993	0.71(0.30–1.67)	0.435
Subgroup 3				
Model 1	0.61(0.32–1.15)	0.125	0.99(0.23–4.28)	0.985
Model 2	0.83(0.43–1.61)	0.586	0.71(0.15–3.41)	0.673
Model 3	0.77(0.40–1.50)	0.443	0.70(0.15–3.39)	0.660
Model 4	0.52(0.24–1.11)	0.092	0.56(0.05–1.45)	0.123

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

3.3. Association between metabolic syndrome and carotid atherosclerosis in individuals with FPG value of <126 mg/dL, 2-h PG value of <200 mg/dL, and no hypertension (subgroup 3)

Among 3904 individuals who underwent OGTT, 2440 (63%) could be assigned to subgroups 3. Their baseline characteristics according to gender are shown in Table 1. Carotid plaque was found in 409 (26%) men and 110 (13%) women and carotid intima-media thickening was found in 202 (13%) men and 69 (8%) women. Unlike subgroups 1 and 2, MetS was not significantly associated with either carotid plaque or intima-media thickening after age adjustment, or even before any adjustment in either gender (Tables 2 and 3).

4. Discussion

Here, we have assessed whether MetS is a risk factor for carotid atherosclerosis in individuals who were determined not to have diabetes mellitus based on results of OGTT. MetS was found to be associated with carotid atherosclerosis especially in men; however, when individuals with hypertension, defined as those having SBP/DBP ≥140/90 mmHg or using anti-hypertensive medication, were excluded, the presence of MetS no longer conferred excess risk when adjustments were made only for age or even when no adjustments were made.

It is known that clustering of certain metabolic abnormalities and hypertension increases the incidence of atherosclerotic diseases [13]. However, whether such clustering of atherogenic risk factors should be separately designated as MetS has been controversial. Whether MetS is independently associated with carotid atherosclerosis has been analyzed in various populations. By analyzing data on a multi-ethnic cohort of apparently healthy individuals in Canada, Paras et al. reported that although MetS was significantly associated with measures of sub-clinical carotid atherosclerosis, this association is mediated entirely through the components of MetS that have been considered as risk factors [14]. Similarly, by analyzing data on individuals recruited from a local community in Italy, Fadini et al. demonstrated that the clustering of MetS components led to a no-more-than additive increase in carotid intima-media thickness [4]. In addition, Vaidya et al. reported that MetS did not have supra-additive association with carotid intima-media thickening [15].

In our previous study that analyzed data on subjects who underwent general health screening, we found that MetS may not be associated with carotid atherosclerosis even after adjustment only for age when individuals did not have hypertension (SBP/DBP <140/90 mmHg and not using anti-hypertensive medication) [8]. In the current study, we expanded this theme to investigate whether MetS increases the risk for carotid atherosclerosis in individuals who had no or only mild (i.e., not in the diabetic range) abnormalities in glucose metabolism. We found that in individuals with FPG values of <126 mg/dL (subgroup 1) or in those with 2-h PG values of <200 mg/dL (subgroup 2), MetS was positively associated with carotid plaque after adjustment for only age (Model 2), although the relationship was only borderline positive in women. In men, the association between MetS and carotid intima-media thickening was also statistically significantly positive after adjustment for only age. These associations lost statistical significance after adjustment for TC, smoking status, and components of MetS (Model 4), suggesting that these associations may not be independent of these factors. Attention should be given to the fact that after excluding individuals with hypertension from the analysis, the association between MetS and carotid plaque or carotid intima-media thickening was no longer statistically significant even after adjustment for only age (subgroup 3), which is in agreement with our previous finding [8].

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