Bone marrow-derived cells fuse with hepatic oval cells but are not involved in hepatic tumorigenesis in the choline-deficient ethionine-supplemented diet rat model

Koji Kubota, Junpei Soeda*, Ryousuke Misawa, Motohiro Mihara, Shiro Miwa, Hirohiko Ise1, Masafumi Takahashi1 and Shinichi Miyagawa

Department of Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matumo, Nagano 390-8621, Japan and 1Department of Organ Regeneration, Institute of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Shinshu University School of Medicine, 3-1-1 Asahi, Matumo, Nagano 390-8621, Japan

*To whom correspondence should be addressed. Tel: +81 263 37 2654; Fax: +81 263 35 1282; Email: jsodea@hsp.md.shinshu-u.ac.jp

Bone marrow cells (BMCs) have been reported to behave as tissue-specific stem cells in some organs and to participate in tumorigenesis. However, the roles of BMCs in hepatic regeneration and carcinogenesis are still unknown. A choline-deficient, ethionine-supplemented (CDE) diet leads to the appearance of oval cells, a type of hepatic progenitor cell, and activates their replication. Furthermore, this type of diet induces preneoplastic nodules and hepatocellular carcinomas (HCCs) derived from oval cell progenitors. The aims of this study were to determine whether oval cells are derived from BMCs and whether preneoplastic nodules or HCCs originate from BMCs in the CDE diet rat model. To clarify the origin of constituent cells in the liver, we transplanted BMCs from green fluorescent protein (GFP) transgenic female rats into male Lewis rats, which were then exposed to a CDE diet to induce hepatocarcinogenesis. Some oval cells showed both donor-derived GFP expression and the recipient-specific Y chromosome, indicating that donor BMCs fused with recipient oval cells. Several preneoplastic nodules (precancerous lesions) identified by their glutathione S-transferase placental (GSTp) positivity were induced by CDE treatment. However, these preneoplastic GSTp-positive nodules were not GFP positive. In conclusion, this study has produced two major findings. First, BMCs fuse with some oval cells. Second, BMC-fused oval cells and BMCs might not have malignant potential in the CDE-treated rat model.

Introduction

The liver has an extraordinary ability to regenerate after injury or resection through proliferation of preexisting mature hepatocytes (1,2). When hepatocyte proliferation is blocked or extensive hepatocyte loss occurs, hepatic progenitor cells are activated to restore the liver mass. Oval cells are one type of candidate hepatic progenitor cell in rodent models. These cells are activated by chemical hepatocarcinogenesis or chemical hepatitis injury and can differentiate to form both hepatocytes and cholangiocytes (3–6). Oval cells have been reported to arise from the canals of Hering, bile ductules or periductal cells in different animal models (6–8), but their precise origin is uncertain. Sell et al. (9) postulated that oval cells, activated by alkylation alcohol or a choline-deficient ethionine-supplemented (CDE) diet, originate from periductal progenitor cells, which may be derived from bone marrow. Petersen et al. (10) reported that bone marrow cell (BMC)-derived oval cells appeared in the liver after treatment with both 2-acetylaminofluorene (2-AAF) and carbon tetrachloride. However, other studies have suggested that BMC-derived cells do not significantly contribute to oval cells (11–14). Recent studies have suggested that BMCs also generate hepatocytes in animal models and human diseased liver (15–18). Thorgeirsson et al. (19) reviewed these papers and advocated that BMCs contribute little to hepatocyte formation under either physiological or pathological conditions and that fusion of BMCs and hepatocytes is the main mechanism by which hepatocytes carrying a bone marrow tag are generated. Thus, the role of BMCs in liver regeneration is still controversial.

In the rodent hepatocarcinogenesis model, hepatocellular carcinoma (HCC) originates from two cellular sources, (i) mature hepatocytes, which respond to diethylnitrosamine (DEN) hepatocarcinogenesis (20), and (ii) hepatic progenitor cells (oval cells), which respond to 2-AAF-based regimens (21) or choline deficiency regimens (22–24). In humans, it has also been suggested that some HCCs are derived from hepatic progenitor cells (25,26). On the other hand, BMCs are reported to develop into gastric cancer in the mouse Helicobacter pylori infection model (27). However, it is unclear whether BMCs contribute to hepatic tumorigenesis.

In the present study, we investigated the involvement of BMCs in hepatic regeneration and carcinogenesis. To clarify the origin of constituent cells of the liver, we transplanted BMCs from green fluorescent protein (GFP) transgenic female rats into male Lewis rats. We used the CDE diet carcinogenesis model, which is thought to be a likely candidate for bone marrow-derived liver stem cells, because we hypothesized that such bone marrow-derived liver stem cells may be the precursors of cancer cells. This study had two goals: (i) to determine whether oval cells are derived from BMCs and (ii) to examine whether preneoplastic nodules or HCCs are originated from BMCs in this model.

Materials and methods

Animals

Lewis rats were purchased from Japan SLC (Shizuoka, Japan). Lewis transgenic rats that ubiquitously express enhanced GFP (GFP transgenic rats) were a generous gift from YS New Technology Institute (Tochigi, Japan) (28,29). The animals were housed at a constant temperature of 18–20°C under a 12 h light/dark cycle. All experiments met the requirements stipulated by Shinshu University Guide for Laboratory Animals.

Bone marrow transplantation and CDE diet treatment

Recipient rats (male 8-week-old Lewis rats) were subjected to 10 Gy total body irradiation using a linear accelerator (MBR-1505RS, Hitachi Medical Corporation, Tokyo, Japan). Briefly, the thigh was removed from a dead donor rat (female GFP transgenic rat), and BMCs were harvested and suspended in phosphate-buffered saline (PBS). The BMCs were then filtered through 70 m nylon mesh (Becton and Dickinson Company, Franklin Lakes, NJ). To destroy any red blood cells, the BMCs were washed in ammonium chloride-potassium phosphate buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM ethylenediaminetetraacetic acid) for 10 min on ice and adjusted to 1 × 106 cells/ml with Dulbecco’s modified Eagle medium. We injected 5 × 107 BMCs via the tail vein of each recipient rat. To confirm successful bone marrow transplantation (BMT), peripheral blood was obtained from the tail vein of each rat, 6 weeks after BMT, and was examined by flow cytometric analysis (FACScalibur; Becton Dickinson and Company) and fluorescence microscopy (BX60 fluorescence microscope; Olympus, Tokyo, Japan).

BMT rats were fed a choline-deficient diet containing 0.1% Nt-ethionine (CDE diet) (Oriental Yeast Co. Ltd, Tokyo, Japan) to induce hepatocarcinogenesis and then killed at 1 (n = 6), 3 (n = 6), 8 (n = 3) and 12 weeks (n = 7) after CDE treatment. Control BMT rats (without a CDE diet) were killed at 1 (n = 4), 3 (n = 4), 8 (n = 3) and 12 weeks (n = 4) after analysis for chimerism (Figure 1A). Non-BMT control Lewis rats were fed the CDE diet for 12 weeks (n = 5).

Histology and immunohistochemistry

Liver tissues obtained from experimental rats were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Three-micrometer thick sections were...
incubated in 0.8% pepsin (wt/vol)/0.2 mol HCl at 37°C for 10 min, followed by quenching with pepsin in 0.2% glycine (wt/vol)/2× PBS and washing in PBS. They were then fixed in 4% paraformaldehyde for 2 min at room temperature and washed three times in PBS over 15 min. Serial ethanol dehydration was done, and the slides were air-dried. A rat Y chromosome probe labeled with fluorescein isothiocyanate (STAR FISH; Cambio, Cambridge, UK) was applied to the sections, which were then coveredslipped and sealed with rubber cement. These sealed slides were denatured at 65°C for 10 min and incubated overnight in a hydrated slide box at 37°C. The coverslips were then carefully removed, and the sections were washed in deionized formamide 50% (wt/vol)/2× standard saline citrate at 37°C three times for 5 min each time, then in 2× standard saline citrate at 37°C three times over 15 min and in 0.05% (wt/vol) Tween 20/4× standard saline citrate at 37°C for 10 min. The slides were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes).

**Quantification of BMC-derived cells**

Quantification of BMC-derived oval cells was performed by counting the GFP and CK 19 double-positive oval cells in five random ×400 images of each liver section taken from rats 1 (n = 6), 3 (n = 6), 8 (n = 3) and 12 weeks (n = 7) after CDE treatment and BMT-only rats. The average numbers of CK 19-positive oval cells, GFP-positive cells and GFP/CK 19 double-positive oval cells in each group were calculated.

To evaluate the proportion of cells double-positive for GFP and the Y chromosome, 12 random ×400 images were counted in each animal from 3 weeks after CDE treatment. Tissue sections from non-treated female rats were used as negative controls (Figure 1A).

A nodule was defined as preneoplastic if >80% of cells in the nodule were positive for GSTp. To calculate the total numbers of nodules and preneoplastic nodules, 20 random ×400 images were counted in each liver section taken from each rat after 12 weeks of CDE treatment with BMT (n = 7) and without BMT (n = 5).

All specimens in this study were analyzed with a BX60 fluorescence microscope (Olympus) or a BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan).

**Statistical analysis**

Values are expressed as the mean ± SD. Statistical significance was evaluated using Student’s t-test or the Kruskal–Wallis test with the Steel–Dwass test. Differences at P < 0.05 were considered to be statistically significant. All statistical analysis was done with the statistical software Microsoft Excel (Microsoft, Redmond, WA) and Excel Toukei (Social Survey Research Information Co., Ltd, Tokyo, Japan).

**Results**

**Analysis of chimerism**

To confirm bone marrow replacement with GFP-expressing cells, peripheral blood cells derived from each BMT rat were assessed by flow cytometric analysis and direct visualization with fluorescence microscopy (Figure 1B). All myeloid and lymphocytic lineage cells of BMT rats showed 93–96% GFP labeling. To evaluate the stability of GFP expression during the entire observation period, BMCs removed from each BMT rat were assessed by direct visualization with fluorescence microscopy. GFP expression was stable during this study (Figure 1C), indicating that BMT had been successful.

**Characterization of liver from CDE diet-fed rats**

Oval cells are a morphologically defined population of small basophilic cells with small ovoid nuclei. These cells have a high nuclear to cytoplasmic ratio. After 1 week of CDE treatment, hepatic steatosis occurred and many ballooned hepatocytes were observed. In addition, a few oval cells were evident, especially around the periportal regions of the liver (Figure 2A and B). After 3 weeks of CDE treatment, accumulation of oval cells around the portal region had become obvious (Figure 2B and E), while at 8 weeks, these cells had spread toward the central vein regions. In contrast, ballooned hepatocytes were decreased (Figure 2G and H). Furthermore, substantial numbers of oval cells with a duct-like morphology appeared throughout the liver after 12 weeks of CDE treatment (Figure 2J and K). There was no obvious histological change in each liver of BMT rats without...
CDE treatment at each of the time points (negative control groups) (Figure 2M and N). In addition, oval cells were evaluated by expression of CK 19, a marker of bile duct epithelial cells and oval cells (30,31) (Figure 2C, F, I, L and O) (Table I). In the BMT groups without CDE treatment (negative control groups), only bile epithelial cells were stained by this antibody (Figure 2O). Immunostaining for OV-6 (32) and CD34 (33), markers of rat oval cells, also confirmed the same result as CK 19 immunostaining (data not shown). These results indicated that BMT alone (including irradiation) did not affect the liver in this study.

Fig. 2. Hematoxylin–eosin and immunohistochemical staining of CK 19 in the CDE-treated liver. (A, B, D, E, G, H, J, K, M and N) Hematoxylin–eosin staining. (C, F, I, L and O) Immunohistochemical analysis of CK 19. (A–C) one week after CDE treatment (n = 6); (D–F) 3 weeks after CDE treatment (n = 6); (G–I) 8 weeks after CDE treatment (n = 3); (J–L) 12 weeks after CDE treatment (n = 7) and (M–O) 18 weeks after BMT without CDE treatment (n = 4). (A, D, G, J and M) Bar = 20 μm. (B, E, H, K and N) Bar = 5 μm; (C, F, I, L and O) bar = 50 μm.

Table I. Quantification of CK 19 and GFP double-positive oval cells

<table>
<thead>
<tr>
<th></th>
<th>Average ± SD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week (n = 4)</td>
<td>3 weeks (n = 4)</td>
<td>12 weeks (n = 4)</td>
</tr>
<tr>
<td>BMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 19(+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP(+)</td>
<td>11.25 ± 1.89</td>
<td>11.00 ± 2.94</td>
<td>9.75 ± 1.26</td>
</tr>
<tr>
<td>GFP and CK 19(+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP and CK 19(+)/CK 19(+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDE + BMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 19(+)</td>
<td>90.67 ± 3.51</td>
<td>953.67 ± 53.49</td>
<td>3098.29 ± 50.47</td>
</tr>
<tr>
<td>GFP(+)</td>
<td>12.67 ± 1.37</td>
<td>103.17 ± 10.23</td>
<td>305.14 ± 13.51</td>
</tr>
<tr>
<td>GFP and CK 19(+)</td>
<td>33.00 ± 5.44</td>
<td>118.57 ± 10.33</td>
<td>3.83 ± 0.32</td>
</tr>
<tr>
<td>GFP and CK 19(+)/CK 19(+)</td>
<td>3.45 ± 0.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CK 19(+) number of CK 19-positive oval cells; GFP(+), number of GFP-positive cells; GFP and CK 19, number of GFP and CK 19 double-positive oval cells; GFP and CK 19(+) / CK 19(+) percentage of GFP and CK 19 double-positive oval cells among CK 19-positive oval cells.

*P < 0.05 versus previous time point
**P < 0.05 versus negative control (BMT without CDE groups).
Bone marrow-derived liver cells

Cells from female donor rats were GFP positive. After 1 week of CDE treatment, GFP-positive cells were not observed in the periportal regions (Figure 3A), but after 3 weeks of CDE treatment, GFP-positive cells were observed (Figure 3B). After 8 and 12 weeks of CDE treatment, GFP-positive cells had spread toward the central vein regions, similarly to the population of oval cells (Figure 3C and D). In the BMT group without CDE treatment, only few GFP-positive cells were evident at each time point (Figure 3E) (Table I). The liver of 12 weeks CDE treatment without BMT was not stained by the anti-GFP antibody (Figure 3F). In contrast, the liver of GFP transgenic rats was extensively stained with this antibody (Figure 3G). To identify GFP-positive (donor derived) cells with oval cells, we performed double immunofluorescence for GFP (green) and CK 19 (red). About 30% of GFP-positive cells were costained with CK 19 antibody, and these double-positive cells were considered to be BMC-derived oval cells (Figure 3H). However, the percentage of GFP-positive oval cells was low and did not differ significantly between rats after 3 and 12 weeks of CDE treatment (Table I), and GFP-positive hepatocytes were not observed.

To determine whether cell fusion is the mechanism that facilitates BMC integration, we assayed for the presence of the Y chromosome in GFP-positive cells in the liver at 3 weeks after CDE treatment. We selected this time point because it coincided with the first appearance of GFP-positive oval cells. Cells from female donor GFP transgenic rats were identified as GFP positive and Y chromosome negative. If BMC-derived oval cells were generated by cellular fusion, then GFP-positive cells must have the Y chromosome. Colocalization of donor-derived GFP expression and recipient-specific Y chromosome labeling within the same cell indicated that donor BMCs had fused with recipient oval cells (Figure 4).

To compensate for undercounting of Y chromosome-positive nuclei due to partial nuclear sampling in tissue sections, cell counts were normalized to the percentage of Y chromosome-positive cells seen in the liver at 3 weeks after CDE treatment. This adjustment was performed on the basis that 3 µm thin sections were analyzed, whereas the nuclear diameter in rat liver is >3 µm. Thus, nuclei are only partially sampled in sections, and some nuclei will not have demonstrable Y chromosomes in these sections. To compensate for this artifact of sectioning, we counted Y chromosomes in all nuclei in the same section. In the liver at 3 weeks after CDE treatment (n = 6), 31.74 ± 0.51% (range: 30.98–32.12%) of all nuclei and 30.25 ± 2.57% (range: 26.09–33.78%) of GFP-expressing cells were Y chromosome positive. Thus, quantitative analysis indicated that 95.31% of GFP-positive cells (3.29% of oval cells) were Y chromosome positive (Table II).

Relationship with BMCs and hepatocarcinogenesis

Before 8 weeks of CDE treatment, nodules were not induced (Figure 5A). However, several macroscopic and microscopic nodules were observed after 12 weeks of CDE treatment (Figure 5B and C). GSTp is known to be the most accurate marker enzyme for detection of initiated cells during liver carcinogenesis (34,35). In agreement with previous reports, some nodules were stained for GSTp, indicating that...
these nodules were preneoplastic lesions (36,37) (Figure 5D). To evaluate the influence of BMT on hepatocarcinogenesis, the number of nodules and preneoplastic nodules after 12 weeks of CDE treatment were compared between the BMT and non-BMT groups, but there were no significant differences between the two groups (BMT, n = 7; versus non-BMT, n = 5); nodules, 228.14 ± 26.77 versus 260.00 ± 50.87, P = 0.25; preneoplastic nodules, 36.71 ± 6.47 versus 38.80 ± 3.90, P = 0.50). In addition, we did not find GFP-positive hepatocytes in any (GSTp positive and negative) nodules (Figure 5E). Furthermore, GSTp and GFP double-positive nodules were not observed in this model (Figure 5F). GFP immunoreactivity was only observed in some oval cell populations, inflammatory cells and sinusoidal lining cells in these nodules. These results suggest that preneoplastic lesions (GSTp-positive nodules) are not derived from BMCs and BMC-derived oval cells. Furthermore, BMCs did not fuse with hepatocytes or transformed (GSTp positive) hepatocytes.

Discussion

Our present study produced two major findings. First, BMCs fused with some oval cells. Second, BMC-fused oval cells and BMCs might not have malignant potential in the CDE-treated rat model.

Petersen et al. (10) first showed that oval cells could be derived from bone marrow in the 2-AAF/carbon tetrachloride rat model. However, other studies have suggested that oval cells are not generated from BMCs (11–14). In this study, some oval cells were GFP positive. One week of CDE treatment after BMT significantly increased the number of oval cells in the liver compared with the number in rats with BMT alone. However, the number of GFP-positive cells in the liver did not differ significantly between the two groups, and no GFP and CK 19 double-positive cells were observed at 1 week after CDE treatment (Table I). GFP-positive oval cells started to appear after 3 weeks of CDE treatment and these cells showed recipient-specific Y chromosome labeling. If BMC-derived cells originated from a direct differentiation process, oval cells would already express the GFP marker at 1 week after CDE treatment. These results strongly indicated that donor BMCs had fused with recipient endogenous oval cells in this model. No difference in the percentage of BMT-fused oval cells between 3 and 12 weeks of CDE treatment and their lack of subsequent differentiation into hepatocytes indicated that BMC-fused oval cells may have no selective advantage. Oh et al. (38) have recently documented that bone marrow-derived cells directly differentiate to oval cells in a model involving 2-AAF/partial hepatectomy and exposure to monocrotaline. The difference between our results and those of Oh et al. is presumably due to the differences in the types of liver injury and inhibition of cell proliferation used. They suggested that two conditions are needed to induce differentiation of hepatic oval cells from bone marrow. First, the liver injury has to be sufficient to activate the oval cell compartment within the liver. The second requirement is inhibition of the proliferation of all resident cell types that could influence and promote liver regeneration. The liver injury caused by the CDE diet is known to induce a high number of oval cells after a few days of treatment (39–41), which satisfies the first requirement but not the second.
In addition, we did not detect bone marrow-derived hepatocytes in this study. It has been reported that 30% of hepatocytes fuse with BMCs in the liver of fumarylacetoacetate hydrolase-deficient mice (42,43). However, in a review, Snorri et al. (19) have considered that BMCs contribute little to hepatocyte formation under either physiological or pathological conditions. These results suggest that hepatocyte fusion with BMCs is rare in traditional liver injury models created by chemical or physical inducers, but is prominent in long-term and contentious liver injury caused by fumarylacetoacetate hydrolase deficiency. No report has yet addressed fusion between hepatocytes and BMCs in the CDE model; thus, it is difficult to evaluate whether the apparent lack of hepatocyte fusion with BMCs we observed here is characteristic of the CDE model.

Fusion between BMCs and oval cells led us to consider whether transplanted BMCs act as tumor stem cells. There is a precedent for the involvement of cellular fusion in tumorigenesis. Bjerkvig et al. (44) have considered that inappropriate cell–cell fusion might contribute to cancer progression. It is intriguing to hypothesize that cellular fusion between BMCs and oval cells triggers hepatic tumorigenesis. Wang et al. (27) argued that chronic infection of C57BL/6 mice with H. pylori induced repopulation of the stomach with BMC-derived cells and that, subsequently, these cells progress through metaplasia and dysplasia to intraepithelial cancer. BMCs at least appear to act as cancer stem cells in the gastric cancer mouse model. To our knowledge, only one report has addressed the relationship between BMCs and hepatocarcinogenesis. Ishikawa et al. (45) reported that BMCs were not the original cell lineage of HCC induced by treatment with DEN and phenobarbital in a mouse model. There are two major hypotheses concerning the origin of HCC: the hepatocyte theory and the stem cell theory. In the DEN treatment model, HCC originates from mature hepatocytes (20), and this mode of carcinogenesis corresponds to the hepatocyte theory. On the other hand, in the CDE model, preneoplastic nodules are induced, and HCC develops from oval cell progenitors (22–24). The carcinogenesis in this model is explained by the stem cell theory, unlike the DEN/phenobarbital model.

However, in the present study, GSTp-expressing preneoplastic nodules were observed in the liver after 12 weeks of CDE treatment, but these nodules were not GFP positive. Furthermore, the number of preneoplastic nodules in rats with and without BMT after 12 weeks of CDE treatment did not differ significantly, and preneoplastic nodules were not induced at 18 weeks after BMT without CDE treatment. These results suggested that BMT (including irradiation) did not influence hepatocarcinogenesis in this study. Rizvi et al. (46) reported that BMCs fused with normal intestinal stem cells and transformed (neoplastic) epithelial cells of the intestine. Cellular fusion between BMCs and intestinal stem cells is not involved in tumor initiation in the small intestine. On the other hand, the observation that BMCs can fuse with transformed (neoplastic) epithelial cells of the intestine is an important finding in terms of the biology of tumorigenesis. Our data suggest that neither BMC-fused oval cells nor BMCs are involved in hepatocarcinogenesis. Moreover, BMCs did not fuse with transformed (GSTp positive) hepatocytes.

Liver transplantation has already been introduced for the treatment of patients suffering from otherwise fatal hepatic diseases, such as

![Fig. 5. Macroscopic findings, hematoxylin–eosin staining, immunohistochemical analysis of GSTp and GFP and double immunofluorescence for GSTp plus GFP analysis in a preneoplastic lesion. (A and C) Hematoxylin–eosin staining. (B) Macroscopic findings. (D) Immunohistochemical analysis of GSTp. (E) Immunohistochemical analysis of GFP. (F) Double immunofluorescence analysis of GSTp (red), GFP (green) and 4′,6-diamidino-2-phenylindole (blue); (A) 8 weeks after CDE treatment (n = 3); (B–F) 12 weeks after CDE treatment (n = 7). (A and C) Bar = 50 μm; (D, E and upper F) bar = 20 μm; (lower F) bar = 5 μm.](https://academic.oup.com/carcin/article-abstract/29/2/448/2526915)
Funding
The Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (B) 17390362] to S.M.

Acknowledgements
We are grateful to Masami Narita for excellent technical assistance. We thank YS New Technology Institute for providing GFP transgenic rats.

Conflict of Interest Statement: None declared.

References

Received July 13, 2007; revised November 7, 2007; accepted December 1, 2007