Inhibitory Effect of Anti-rheumatic Drug Iguratimod for Hepatocellular Carcinogenesis by Inhibition of Serum Interleukin-8 Production

TARO SAKAMOTO, YUJI ISHII, HIROAKI SHIBA, KENEI FURUKAWA, YUKI FUJIWARA, KOICHIRO HARUKI, RYOTA IWASE, YOSHIHIRO SHIRAI and KATSUHIKO YANAGA

Department of Surgery, The Jikei University School of Medicine, Tokyo, Japan

Abstract. *Background/Aim: Angiogenesis is a known factor for the development of hepatocellular carcinoma (HCC). The aim of this study was to assess the property of iguratimod, that is an anti-inflammatory drug for rheumatoid arthritis, on anti-angiogenesis and anti-carcinogensis for HCC. Materials and Methods: In vitro, human umbilical vein endothelial cells were cultured under interleukin-8 (IL-8) with or without iguratimod. In vivo, a rat model with HCC received iguratimod or distilled water for 6 weeks. Diameter of the largest tumor, number of tumors and serum interleukin-8 concentration were compared between iguratimod and control groups. Results: By an in vitro angiogenesis assay, it was found angiogenesis in iguratimod group was significantly lower than that in control group (p=0.013). In vivo, largest tumor diameter (p=0.036), number of the tumor (p=0.011) and serum interleukin-8 concentration (p=0.036) in the iguratimod group were significantly smaller and lower than those in the control group. Conclusion: Iguratimod may inhibit hepatocellular carcinogensis by inhibition of interleukin-8 production in a rat model.*

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies in the world. Hepatic resection is a potentially curative treatment of HCC. Although operative mortality of elective hepatic resection has been minimized by improvements in surgical techniques, instruments and perioperative managements (1), the incidence of recurrent HCC after hepatic resection remains as high as 60-70% (2). Therefore, preemptive treatment before development of HCC for high-risk patients for viral hepatitis and non-alcoholic steatohepatitis (NASH) that are related to chronic inflammation (3, 4), as well as adjuvant therapy after hepatic resection for HCC, could improve therapeutic outcome of HCC.

Chronic hepatitis viral infection with consequent inflammatory immune response has an important role in carcinogenesis and development of HCC (5-8). Pro-inflammatory cytokines have been associated with chronic inflammation and increased levels of cytokines induce carcinogenesis (9). Moreover, interleukin (IL)-8 induces angiogenesis through activation of the vascular endothelial growth factor (VEGF) pathway and enhances the metastatic activity of malignancies (10). Inhibition of cytokines, including IL-8 production, may prevent carcinogenesis, which could be a potential treatment of malignancies (11, 12).

Iguratimod is an anti-inflammatory drug for the treatment of rheumatoid arthritis (13, 14) that prevents the production of inflammatory cytokines, including tumor necrosis factor (TNF)-alpha, IL-6 and IL-8 by the suppression of nuclear factor-kappa B (NF-KB) (15, 16). However, to the best of our knowledge, anti-carcinogenesis and anti-angiogenesis of iguratimod for malignancies, including HCC, has not been investigated.

The aim of this study was to assess if hepatocellular carcinogenesis and IL-8-induced angiogenesis is inhibited by Igratimod *in vitro* and *in vivo*.

Materials and Methods

Iguratimod. Iguratimod, an anti-inflammatory drug for the treatment of rheumatoid arthritis (13, 14), was a generous gift from Toyama Chemical Co. Ltd. (Tokyo, Japan).

Angiogenesis assay in vitro. An *in vitro* angiogenesis assay kit (Kurabo, Osaka, Japan) was used according to the manufacturer's instructions (17-20). Briefly, as study groups, human umbilical vein endothelial cells (HUVEC) co-cultured with human fibroblasts, were cultured with 100 ng/ml of human IL-8 recombinant protein

Correspondence to: Taro Sakamoto, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan. Tel: +81 334331111 (ext. 3401), Fax: +81 354724140, e-mail: sakamo@jikei.ac.jp

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together with or without iguratiomd (5 μg/ml) for 10 days. As control group, HUVEC co-cultured with human fibroblasts were cultured without IL-8 or iguratimod and with 5 μg/ml of iguratimod alone, thus constituting the iguratimod group. The medium was changed every 3 days. Cells were fixed in 70% ethanol and then visualized with an anti-CD31 antibody according to the protocol. CD31-positive area was measured quantitatively using Kurabo angiogenesis image analyzer (Kurabo, Osaka, Japan) in five different fields for each condition, under microscopy.

Induction of hepatcellular carcinoma. Five-week-old male Fisher rats were housed in plastic cages with paper chip bedding (Alpha-dri; Shepherd Specialty Papers Inc., Michigan, USA) in a biological cabinet at the Laboratory Animal Facilities of the Jikei University School of Medicine. The animals were maintained during the experiment on a 12-hour light-dark cycle at a temperature of 22±2˚C and 55±5% humidity in a room with a filtered air supply. An acclimation period of 4 days was allowed. The animals received free drinking water with 100 mg/l of diethylnitrosamine (DEN) (Sigma Chemical Co., St. Louis, MO, USA) for 12 weeks. HCC induced in rats by DEN, fed *via* a tumor-specific artery, is reported to be hypervascular (21-23).

At the end of the induction period, animals were sacrificed to check for tumor formation. Tumors were found only in the liver where multiple tumor nodules were identified in each of the 3 animals. The largest tumor size of each rat ranged from 3 to 8 mm, while the number of tumors ranged from 8 to 35.

The animal protocol described in this study was approved by the Laboratory Animal Facilities of the Jikei University School of Medicine and performed in accordance with the National Institutes of Health Guidelines.

Iguratimod treatment models in vivo. For the iguratimod group (n=4), the animals that have received DEN for 10 weeks also received 5 mg/kg/day of iguratimod diluted with sterilized 0.5 w/v% methyl cellulose 400cP solution (Wako Pure Chemical Industries, ltd., Osaka, Japan), 5 days per week for 6 weeks. Control group (n=3) of the animals with HCC induced by DEN received solvent alone for 5 days per week for 6 weeks.

At the end of the study period, the animals were sacrificed and the whole liver was excised. The diameter of the largest tumor was measured and the tumor number was counted in each animal. Serum IL-8 concentration was measured at the time of sacrifice. The specimens for immunohistochemical staining with CD31, obtained from tumor and non-tumor areas of the liver, were paraformaldehyde-fixed and paraffin-embedded. CD31 immunohistochemical staining was performed by Ventana Japan K.K. (Yokohama, Japan).

Statistical analysis. The data were expressed as a mean±standard deviation (SD). The non-paired Student's *t*-test and non-parametric Mann-Whitney's *U*-test were used for statistical studies. All *p*-values were considered statistically significant when the associated probability was less than 0.05.

Results

Inhibition of interleukin-8-induced angiogenesis by iguratimod in vitro. Figure 1 demonstrates immunohisto-chemical staining for CD31. CD31-positive cells were stained as black areas, which were more frequent in IL-8 group (Figure 1C) as compared to those in control (Figure 1A), iguratimod (Figure 1B) or the IL-8 treated with iguratimod group (Figure 1D). Figure 2 shows quantitative analysis of the results of angiogenesis assay. CD31-positive area in iguratimod group (46,860.8±16,391.5; p=0.875) was similar to that in control group (47,684.4±16,513.1 pixels; mean±SD). CD31-positive area in the IL-8 treated with iguratimod group (43,579.1±15,860.3) was significantly smaller than that in IL-8 group (57,717.9±18.971.2; p=0.013).

Inhibition of hepatocellular carcinogensis and interleukin-8 production by iguratimod in vivo. Figure 3 shows excised livers of control (Figure 3A) and iguratimod groups at sacrifice (Figure 3B). Multiple hepatic tumors were shown in the cirrhotic livers of both groups. The diameter of the largest tumor in iguratimod group (7.3±3.1 mm) was significantly smaller than that in control group (13.0±1.7; p=0.036, Figure 4A). The number of tumors in iguratimod group (15.3±4.6) was significantly less than that in control group (32.0±6.6; p=0.011, Figure 4B). The serum IL-8 concentration of iguratimod group (125.3±85.1 ng/ml) was significantly lower than that in control group (275.0±42.4; p=0.036, Figure 4C).

CD31 immunohistochemical staining. Figure 5 demonstrates immunohistochemical staining for CD31 of excised liver specimens at sacrifice. CD31-positive vascular endothelial cells were stained as bright brown areas in sections. In iguratimod treatment group (Figure 5B), CD31-positive cells tended to be fewer than that in control group (Figure 5A).

Discussion

Alteration of various cytokines' expression in serum and liver tissue in patients with HCC have been investigated using quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay and immunohistochemistry (5). T helper cell (Th) 2-like cytokine profile, including an increase in IL-4, IL-8, IL-10 and IL-5, as well as a decrease in Th1-like cytokines, including IL-1α, IL-1β, IL-2, IL-12p35, IL-12p40, IL-15, TNF-α and interferon (IFN)-γ, is associated with the metastatic phenotype (24). Accumulated evidence indicated that immune cells in tumor-surrounding tissue influence tumor progression (25). Antitumor effects of cytokine-based HCC treatments have been reported, including adenovirus vector-mediated IL-12 therapy in a HCC mouse model (26), combined immunotherapy with IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in an immunogenic mouse model (27), as well as IL-2/IFN-α2b fused gene therapy for α-fetoprotein-expressing HCC cells (28). These results suggested that cytokine-based HCC treatments may become a new clinical therapeutic option.

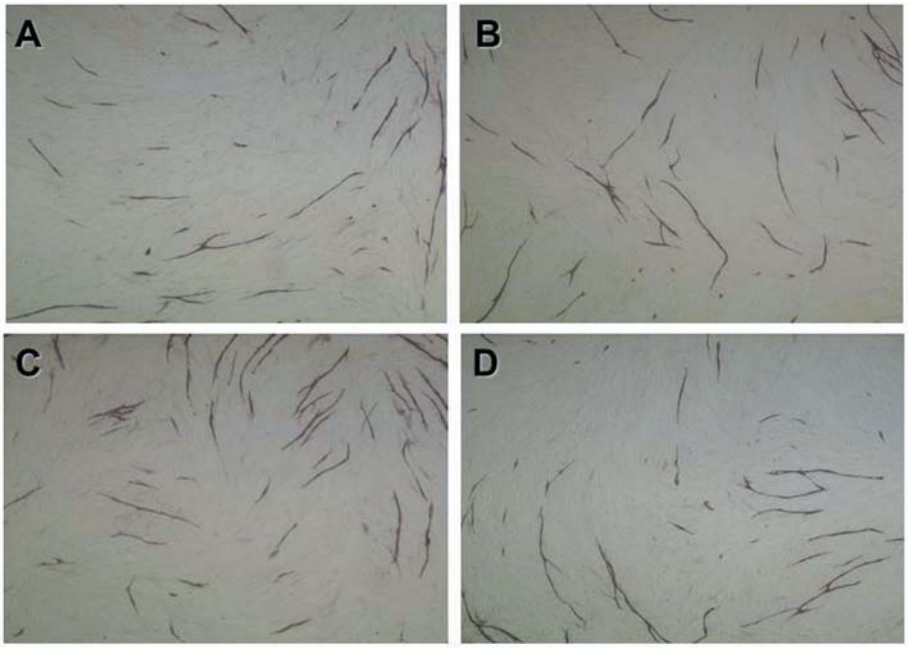

Figure 1. *CD31-positive cells in interleukin-8 group (C) were more frequent than those in control (A), iguratimod (B) or interleukin-8 treated with iguratimod group (D) by angiogenesis assay.*

Cytokines promote induction of inflammatory leucocytes. With recent accumulation of evidence, cytokines and their receptors seem to play key roles in the processes of carcinogenesis, such as autonomous growth signaling, that influence tumor growth, invasion and metastasis (29, 30). Chen *et al*. reported that several cytokines, including fibroblast growth factor 2 (FGF-2), growth-regulated oncogene (GRO), IL-8, interferon gamma-induced protein 10 (IP-10), VEGF and interferon alpha-2 (IFN-a2), were significant prognostic predictors of disease-free and overall survival after hepatic resection for HCC and concluded that these cytokines may be suitable for cytokine-targeted therapies for HCC (31).

IL-8 is a proinflammatory multifunctional CXC chemokine, which affects neutrophil chemotaxis, enzyme release and expression of surface adhesion molecules (32, 33). IL-8 may be a significant regulatory factor of tumor microenvironment, including proliferation, angiogenesis and tumor migration of tumor and vascular endothelial cells. Ren *et al*. reported that the preoperative serum IL-8 levels in patients with HCC are significantly higher than those in healthy adults, which positively correlates with tumor size, pathological tumor-node-metastasis stage and disease-free survival after hepatic resection for HCC, and concluded that the preoperative serum IL-8 may be a useful marker of tumor invasiveness and an independent prognostic predictor in patients with HCC (34). Akiba *et al*.

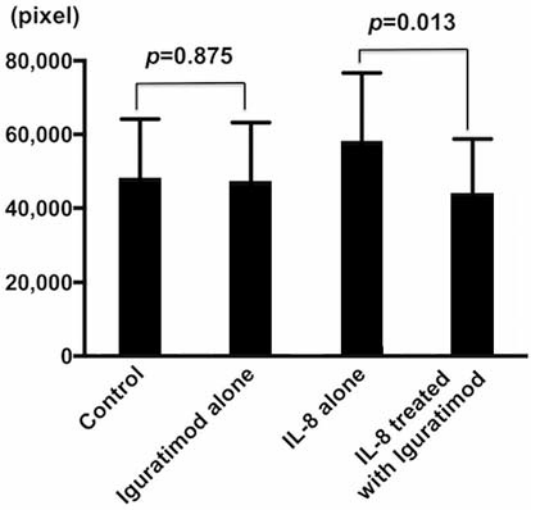

Figure 2. *CD31-positive area in control group was similar to that in iguratimod group (p=0.875). CD31-positive area in the interleukin-8 treated with iguratimod group was significantly smaller than that in the interleukin-8 group (p=0.013).*

indicated that IL-8 regulates tumor cell growth and metastasis in the liver (10). Also, others reported that down-regulation or neutralization of IL-8 inhibited angiogenesis and tumor growth in several tumor models

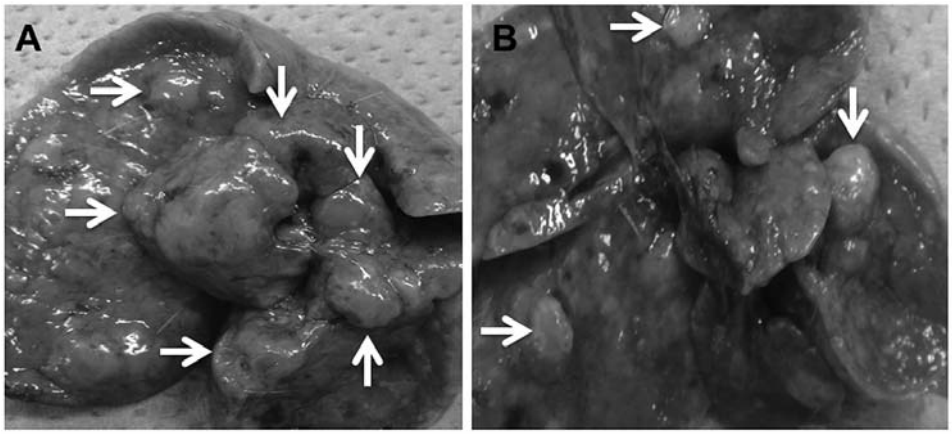

Figure 3. *Multiple hepatic tumors (arrows) are shown in the excised cirrhotic livers of control group (A) and iguratimod group (B) at sacrifice.*

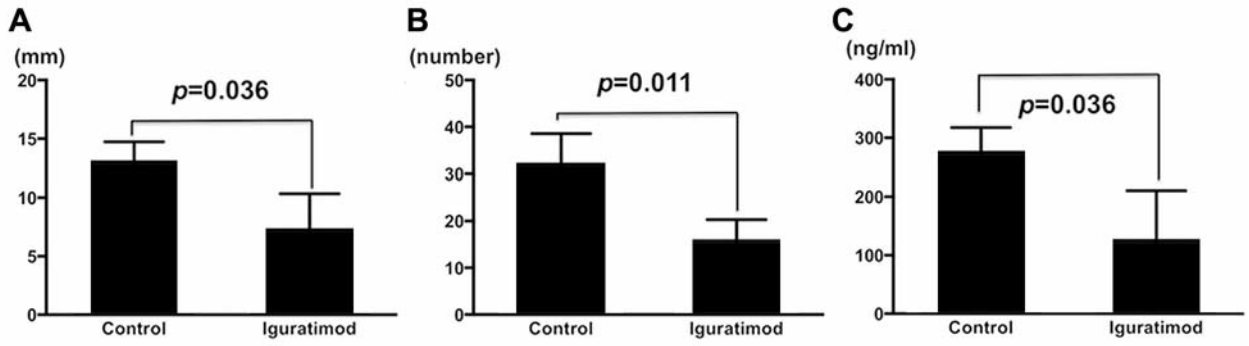

Figure 4. *The diameter of the largest tumor in the iguratimod group was significantly smaller than that in the control group (p=0.036, A). The number of tumors in the iguratimod group was significantly lower than that in the control group (p=0.011, B). The serum interleukin-8 concentration of the iguratimod group was significantly lower than that in the control group (p=0.036, C).*

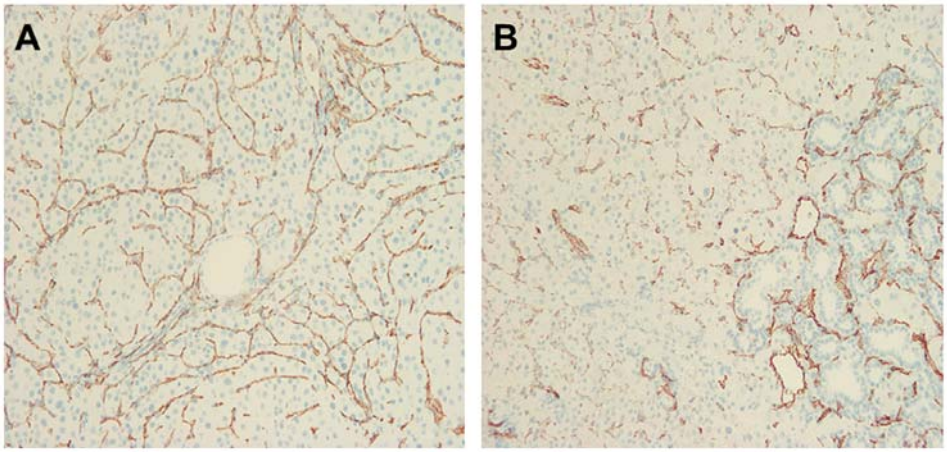

Figure 5. *By CD31 immunohistochemical staining of excised liver specimens, CD31-positive cells in the iguratimod treatment group (B) tended to be fewer than those in the control group (A).*

(11, 12). Shin *et al*. reported that most hepatitis B virus-associated HCC lines produced IL-8 (35). Moreover, serum IL-8 was elevated by ischemia/reperfusion during hepatic resection in patients with HCC (36). These results suggested that hepatic resection may have potent effects on angiogenesis and metastasis, thus indicating that inhibition of IL-8 production may possibly be a preemptive treatment before the development of HCC and adjuvant therapy after hepatic resection for HCC.

Iguratimod, a member of the methanesulfoanilide class of anti-inflammatory agents, is a drug for the treatment of rheumatoid arthritis. Iguratimod inhibits the production of TNF-a, IL-6 and IL-8 in lipopolysaccharide-stimulated THP-1 cells, which is a human monocytic leukemia cell line, by transcriptional regulation through suppression of NF-kB activation (15). Our report and these results suggest that iguratimod inhibits hepatocellular carcinogenesis by suppression of IL-8 production. However, one adverse effect of iguratimod is liver dysfunction, which mainly occurred between 4 and 8 weeks after starting iguratimod (13). In 35 (9.8%) of 377 patients, serum alanine aminotransferase concentration increased to 100 IU/l or higher, while, in 26 patients (6.9%), serum aspartate aminotransferase increased to 100 IU/l or higher. Therefore, patients' selection and dosage of iguratimod investigation are necessary before clinical trials for patients with HCC.

In conclusion, iguratimod seems to prevent hepatocellular carcinogenesis by inhibition of IL-8 production in an experimental rodent model. This anti-inflammatory drug may potentially be a new clinical therapeutic option for HCC.

Disclosure

The Authors declare no conflicts of interest or funding.

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