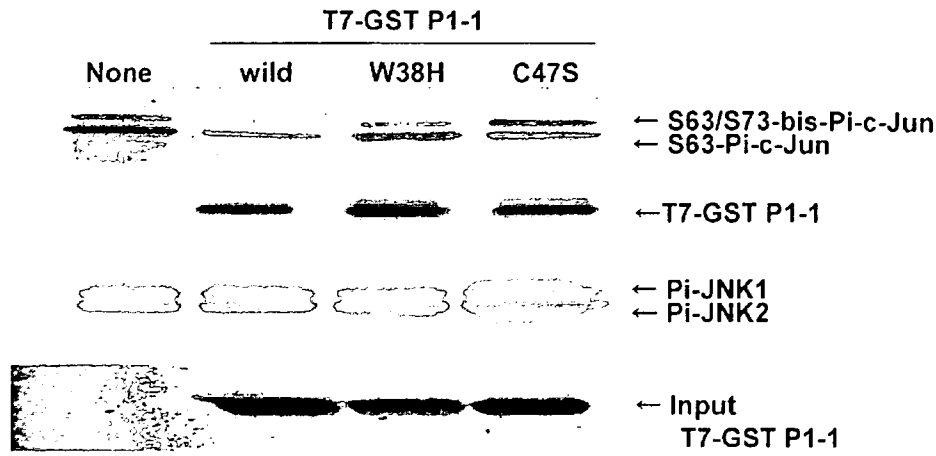

Fig. 5 Inhibitory effect of GST P1-1 (T7-GST P1-1/wild) and its mutants (T7-GST P1-1/W38H and T7-GST P1-1/C47S) on JNK activity. Extracts from AH66 cells irradiated with UV were used as the enzyme source. JNK (including the active form) purified by affinity precipitation (binding to c-Jun fusion resin) was reacted with 100 μM ATP in the presence or absence of T7-GST P1-1/wild or the mutants. After washing the resin, Pi-c-Jun, T7-GST P1-1 and Pi-JNK were measured by Western blot analysis using anti-phospho-c-Jun (63S), anti-T7 and anti-phospho-JNK (183T and 185Y) antibodies, respectively

of that of T7-GST P1-1/wild). By contrast, GST activity of T7-GST P1-1/W38H and T7-GST P1-1/C47S which were site-directed mutations of the active center, was markedly decreased to 8.3 and 1.7% of that of T7-GST P1-1/wild, respectively.

Modification of active center of GST P1-1 influences on JNK activity

To confirm the regulation of JNK activity by GST P1-1, binding of GST P1-1 to the JNK molecule and inhibition of JNK activity by GST P1-1 were determined in the *in vitro* experiment. When affinity-purified JNK including Pi-JNK (active form of JNK) was incubated with recombinant T7-GST P1-1/wild (10 μg/ml) at 30°C for 30 min, T7-GST P1-1/wild was precipitated with the JNK molecule, and potently inhibited the activity of JNK (phosphorylated activity of c-Jun, Pi-c-Jun) in correspondence with T7-GST P1-1/wild binding, but no change in Pi-JNK was observed (Fig. 5). In order to investigate whether or not the active center of the GST P1-1 molecule was required to suppress JNK activity, we used two kinds of site-directed mutated GST P1-1, in which the GSH-binding site residue (G-site) 38W and the substrate-binding site residue (H-site) 47C were replaced with H (T7-GST P1-1/W38H) and S (T7-GST P1-1/C47S), respectively. Although T7-GST P1-1/W38H and T7-GST P1-1/C47S also bound to JNK to a similar degree as T7-GST P1-1/wild (Fig. 5, T7-GST P1-1), these mutants failed to inhibit the activity of JNK (Fig. 5, Pi-c-Jun). Therefore, it was predicted that binding of GST P1-1 to the enzymatically active form of the JNK molecule might be necessary for suppression of JNK activity.

Re-activation of JNK activity by enzymatically inhibited GST P1-1 with GSH-DXR

Since we demonstrated that site-directed mutated GST P1-1 of the active center region failed to inhibit JNK activity, we investigated whether or not inhibition of GST P1-1 activity by GSH-DXR re-activated JNK without dissociation of GST P1-1-JNK complex. We have already reported that GSH-DXR exhibited reverse-inhibition of GST P1-1 activity by binding to the active center (both the G- and H-sites) of the molecule resulting in the induction of apoptosis. By the addition of 10 μM GSH-DXR to the reaction mixture, phosphorylation activity of c-Jun by JNK, which had been inhibited by binding of T7-GST P1-1/wild, was restored (Fig. 6, Pi-c-Jun), but no change in T7-GST P1-1/wild-binding to JNK was observed (Fig. 6, T7-GST P1-1). This result showed that the inhibition of GST P1-1 activity by GSH-DXR re-activated the suppressed JNK activity via the binding of T7-GST P1-1/wild without T7-GST P1-1-liberation from the JNK molecule.

Activation of JNK in AH66 cells transfected with Flag-GST P1-1/W38H and Flag-GST P1-1/C47S by treatment with GSH-DXR

We investigated here whether site-directed mutated GST P1-1 of the active center region failed to inhibit JNK activity in AH66 transfectant cells expressed with Flag-GST P1-1/W38H and Flag-GST P1-1/C47S. Treatment of these transfectant cells with 0.3 μM GSH-DXR for 18 h increased slightly in caspase-3 activation compared with that

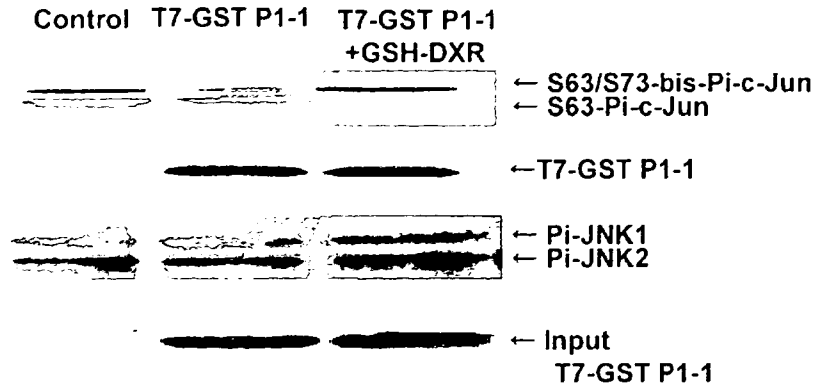

Fig. 6 Inhibition of JNK activity by binding of T7-GST P1-1/wild to JNK and re-activation of the inhibited activity by GSH-DXR. Suspension of JNK-coupled c-Jun fusion beads in kinase buffer (25 mM Tris/HCl (pH 7.5) containing 0.1 mM Na_3VO_4) was incubated with 10 μg/ml T7-GST P1-1/wild at 30°C for 30 min. After washing resin with kinase buffer, T7-GST P1-1/wild-binding JNK was incubated with 100 μM ATP at 30°C for 30 min in the presence or absence of 10 μM GSH-DXR. Pi-c-Jun, T7-GST P1-1/wild and Pi-JNK were measured by Western blot analysis using anti-phospho-c-Jun (63S), anti-T7 and anti-phospho-JNK (183T and 185Y) antibodies, respectively

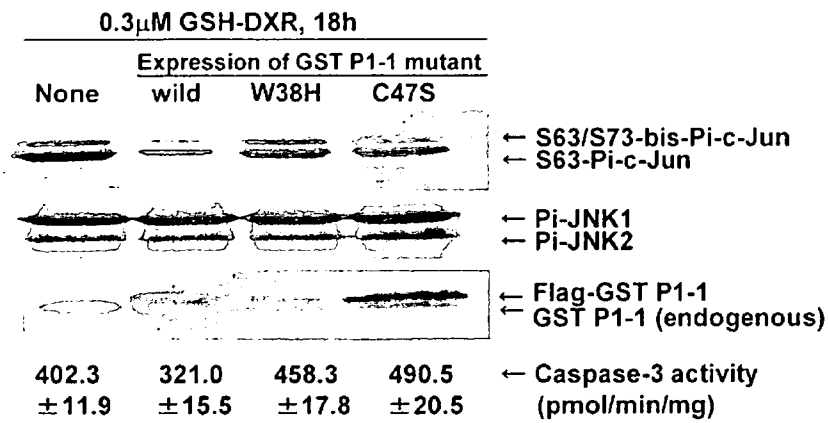

Fig. 7 Activation of JNK in AH66 cells expressed with Flag-GST P1-1/wild, Flag-GST P1-1/W38H and Flag-GST P1-1/C47S by treatment with GSH-DXR. Activation of JNK (phosphorylation of c-Jun and phosphorylated JNK), binding of expressed Flag-GST P1-1/wild, Flag-GST P1-1/W38H and Flag-GST P1-1/C47S to the JNK molecule and activity of caspase-3 in AH66 transfectant cells treated with 0.3 μM GSH-DXR for 18 h were measured. Pi-JNK: phosphorylated JNK (active form of JNK), Pi-c-Jun: phosphorylated c-Jun (phosphorylation of c-Jun by active JNK), GST P1-1: endogenous GST P1-1 and expressed Flag-GST P1-1/wild, Flag-GST P1-1/W38H and Flag-GST P1-1/C47S bound to the JNK molecule. These proteins were analyzed by Western blot analysis using anti-phospho-JNK (183T and 185Y), anti-phospho-c-Jun (63S) and anti-GST-π, respectively. Caspase-3 activity was determined using DEVD-MCA as a substrate

of non-transfectant cells (Fig. 7) although the same treatment of AH66 cells transfected with Flag-GST P1-1/wild decreased in the activation in comparison to that of non-transfectant cells. Moreover, the treatment of the transfectant cells of the active center mutant and the wild-type caused increase and decrease, respectively in the phosphorylated c-Jun level, reflecting higher JNK activity than that of non-transfectant cells. However, the amount of phosphorylated JNK was at the same level in both the transfectant and non-transfectant cells (Fig. 7). Interestingly, expressed Flag-GST P1-1/W38H and Flag-GST P1-1/C47S were also co-precipitated with the JNK molecule and these co-precipitated mutants showed a slight decrease by treatment with GSH-DXR (Fig. 7).

A role of C-terminal region of T7-GST P1-1 on JNK activity

Since it was reported that the C-terminal region of GST P1-1 interacted with the JNK molecule, we investigated the interaction between JNK and the C-terminal mutated GST P1-1. The mutant used for the experiment was that the C-terminal region (194 to 209 amino acids) was deleted (T7-GST P1-1/ΔC(194–209)) and 201R in the region was replaced with A (T7-GST P1-1/R201A). Both T7-GST P1-1 mutants of the C-terminal region, T7-GST P1-1/ΔC(194–209) and T7-GST P1-1/R201A failed to bind and inhibit JNK (Fig. 8). In fact, since T7-GST P1-1/R201A maintained GST activity to the same degree as T7-GST P1-1/wild despite the lack of

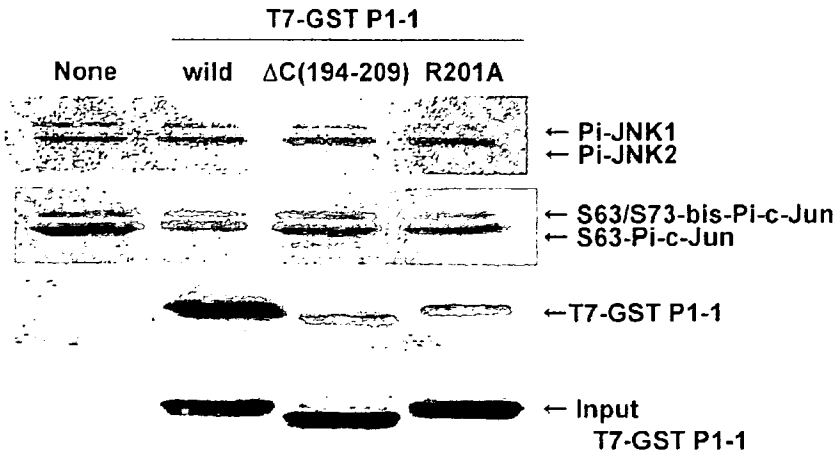

Fig. 8 Effect of C-terminal deletion mutant of GST P1-1 (T7-GST P1-1/ΔC(194–209)) and C-terminal mutated GST P1-1 (T7-GST P1-1/R201A) on binding and activity of JNK. JNK activity was expressed as Pi-c-Jun (phosphorylation of c-Jun by active JNK). Extracts from AH66 cells irradiated with UV were used as the enzyme source. JNK (including the active form) purified by affinity precipitation (binding to c-Jun fusion resin) was reacted with 100 μM ATP in the presence or absence of T7-GST P1-1/wild or the mutants. After washing the resin, Pi-c-Jun, Pi-JNK (active form of JNK), JNK and T7-GST P1-1 were measured by Western blot analysis using anti-phospho-c-Jun (63S), anti-phospho-JNK (183T and 185Y) and anti-T7 antibodies, respectively

binding ability to JNK, T7-GST P1-1/R201A failed to suppress JNK activity.

Discussion

We demonstrated here that the active center (enzyme activity) of intact (full length) GST P1-1 also played an important role in the suppression of JNK activity by binding of GST P1-1 using the mutant form of the active center region, although it was reported that peptide 34–50 of GST-pi containing the active center region suppressed JNK activity without direct binding to JNK [6–9]. Moreover, GSH-DXR-induced apoptosis in AH66 cells caused JNK activation via the suppression of GST P1-1 activity by GSH-DXR.

Our recent study demonstrated that the thiol group of GSH-DXR played an important role in the elevation of cytotoxicity and that the conjugate showed potent inhibition of GST activity and suppression of GST P1-1 expression (mRNA and protein), although DXR did not [25]. Additionally, treatment of AH66 cells with GSH-DXR potently induced DNA fragmentation via caspase-3 activation as compared with DXR [24]. This prompted us to predict that inhibition of GST activity and suppression of GST P1-1 expression by GSH-DXR may be involved in the apoptosis cascade.

An attempt was made to identify a specific downstream signaling molecule regulated by GST P1-1 for the induction of apoptosis in AH66 cells. Recent reports indicated that the binding of GST P1-1 to JNK itself regulated JNK activity and the activation of JNK by apoptosis was induced by certain stresses [6–9]. Therefore, we investigated whether or not JNK was activated by treatment of the cells with GSH-DXR and whether or not JNK activation induced apoptosis. Treatment of AH66 cells with 0.3 μM GSH-DXR caused a continuous increase in JNK activity after 6 h of treatment, however the same treatment resulted in slight dissociation of endogenous GST P1-1 bound to the JNK molecule. It was suggested that the decrease of endogenous GST P1-1 in cells treated with GSH-DXR was dependent upon the amount of JNK-binding GST P1-1. GSH-DXR-induced apoptosis occurred through the mitochondrial pathway, and this reaction was suppressed by approximately 64% as the result of co-treatment with 5 μM SP600125, an inhibitor of JNK. However, caspase-8 but not caspase-12 was activated by the treatment. Therefore, GSH-DXR-induced apoptosis may be carried out through both the mitochondrial pathway and membrane death domain. At least, JNK activation must be induced via apoptosis caused by treatment with GSH-DXR. Although GSH-DXR possessed other apoptosis-inducing pathways, including those inhibiting topoisomerase II activity such as, peroxidation of membrane lipid, and calpain activation, an adequate mechanism of the conjugate has not yet been found to explain the occurrence of potent apoptosis (unpublished data). These findings suggested that the activation of JNK in cells treated with GSH-DXR mainly caused apoptosis *via* the mitochondrial pathway [34–36]. Several reports demonstrated that the transient activation of JNK led to cell proliferation or differentiation and that prolonged JNK activation caused apoptosis [10–14, 43–45]. Moreover, strong and prolonged activation of JNK has been reported in response to lethal doses of a variety of stresses including UVC, γ-radiation and cisplatin, any one of which triggers apoptosis [10, 11, 13, 14, 45]. In our experiment, T7-GST P1-1/wild was able to bind to JNK and decreased JNK activity.

Both T7-GST P1-1/W38H and T7-GST P1-1/C47S bound also to JNK, but these mutants had little inhibitory effect on the activity of JNK. Although T7-GST P1-1/W38H had lost GST activity, the mutant partially inhibited JNK activity. It is therefore more likely that it was not the catalytic efficiency of GST P1-1, but the presence of an intact flexible loop that was necessary for JNK inhibition. This notion is supported by the report of Adler et al. indicating that a flexible loop of GST-pi active center region is important for inhibiting JNK activity [8, 9] using a peptide of the region.

These results suggested that the suppression of GST P1-1 expression and the inhibition of GST P1-1 enzyme activity in cells treated with GSH-DXR potently induced apoptosis via JNK activation. They also suggested that sustained activation of JNK *via* GST P1-1 suppression resulting from treatment with GSH-DXR might induce apoptosis and that the active center region of GST P1-1 was also important in the inhibition of JNK activity. In fact, these enzyme active center-directed mutants that lost their GST enzyme activity also bound to JNK to the same extent as the wild-type, however, both mutants failed to inhibit JNK activity. Moreover, it was confirmed in the AH66 cell line overexpressing GST P1-1 and its mutant that binding of Flag-GST P1-1/W38H and Flag-GST P1-1/C47S to JNK could not even partly inhibit the activity of JNK; and in fact, slightly increased the induction of apoptosis in AH66 cells transfected with GST P1-1 mutated by treatment with GSH-DXR. These findings suggested that the existance of endogenous GST P1-1 weakened the effect of the expressed GST P1-1 mutant on JNK inhibition.

We have reported that GSH-DXR showed noncompetitive inhibition of GST activity [42], meaning that it is an allosteric inhibitor that does not bind to the active-site directly. It is much more likely that GSH-DXR binds directly to the flexible loop region, thereby preventing its inhibitory action on JNK, which is an interesting effect of the conjugate. We also showed that GSH-DXR activated the enzyme activity of JNK without any dissociation of GST P1-1 from the complexed JNK. These results showed that GSH-DXR bound to the GST P1-1 molecule and not to the active center and C-terminal JNK-binding regions. In this experiment, the concentration of GSH-DXR that is known to show 90% inhibition of GST P1-1 activity, 10 μM [26], was used for the assay of JNK activity, since the intracellular GSH-DXR concentration was estimated to be approximately 10 μM when the cells were incubated with 0.3 μM GSH-DXR for 24 h as described in previous reports [24]. Therefore, our results predicted that conformational change in the structure of GST P1-1 caused by the binding of GSH-DXR, re-activated the suppressed JNK catalytic kinase domain or its active site (ATP-binding site on JNK), even though GST P1-1 bound to JNK. As protein-protein interactions leading to the formation of protein complexes are thought to be critical for the normal function of the JNK signaling pathway, conformational change in the binding partner, GST P1-1, is one of the key events in modulation of the JNK enzyme activity. GSH-DXR-treatment suppressed the expression of GST P1-1 although GST P1-1 (mRNA and protein) was transiently expressed at 6 h after treatment [26]. This result suggested that the transient expression of GST P1 mRNA was induced by accelerated translation through the activation of c-Jun via JNK activation. However, findings have suggested that a sustained activation of JNK via GST P1-1 suppression by treatment with GSH-DXR might cause induction of apoptosis through mitochondrial damage; moreover, enzyme activity of GST P1-1 was also found to be an important factor in inhibiting JNK activity. It was also reported that the transient activation of JNK led to cell proliferation or differentiation, but prolonged JNK activation caused apoptosis [12]. Moreover, strong and prolonged activation of JNK has been reported in response to lethal doses of a variety of stresses including UVC, γ radiation and cisplatin, any one of which triggers apoptosis [10–14]. Therefore, the suppression of GST P1-1 expression may be effective in preventing GST P1-1-dependent drug-resistance in several organs.

Demonstration of the molecular interaction was reported, but it was shown that the C-terminal region of GST P1-1 linked indirectly with JNK through competitive binding of the restricted polypeptide of the C-terminal region of the GST P1-1 molecule, which thereby interfered with direct binding between JNK and GST P1-1 [7–9]. Since the positively charged sequence contained residues 194–201 of human GST-pi corresponding to residues 194–209 in rat GST P1-1 in the present study, it was expected that the C-terminal region of GST P1-1 must bind to the negatively charged C-terminus of JNK [6–9]. In the present study, using the deletion mutant of the C-terminal region (T7-GST P1-1/ΔC(194–209)) as well as the site-directed mutant of the same region (T7-GST P1-1/R201A) of GST P1-1, we clarified that the C-terminal region of GST P1-1 binds to JNK. This indicates that the association of GST P1-1 with JNK molecule-regulated JNK activity and that the C-terminal region of GST P1-1 was essential for binding to JNK. It was consisted with the result by Adler et al. [8, 9]. It has been described that Hsp72 has a polypeptide-binding domain of the C-terminal region [46, 47], and binding of the domain to JNK inhibits the activity of JNK [15]. Although GST P1-1 does not have polypeptide-binding domain like Hsp72, the C-terminal region is important for exhibiting the activity that forms a homodimer [19]. It is suggested that the C-terminal region of GST P1-1 binds to a specific peptide domain like the C-terminal region of JNK. It was also reported that GST P1-1 and Hsp72 bound to a C-terminal fragment (200–424) of JNK [7–9, 15]. Since both the ATP-binding site (55K) and phosphorylation sites (183T and 185Y) [48] were not contained in the fragment, it was presumed that binding of

GST P1-1 to JNK exhibited an allosteric inhibitory effect on kinase activity. It was also possible that other sites, such as the N-terminal fraction or active center of the GST P1-1 molecule, disturbed the expression of JNK activity. As mentioned in previous reports, the polypeptide-binding domain (436–618) of Hsp72 binds to JNK and inhibits JNK activity, whereas both the ATP-binding region (120–428) and the N-terminal region (1–120) of Hsp72 does not bind to JNK nor inhibit JNK activity [15]. Therefore, it may be important for the suppression of JNK activity that the active center of GST P1-1 interferes with the ATP-binding site of JNK.

Further study will attempt to identify a specific signaling pathway that plays a role in GSH-DXR-induced apoptosis.

Conclusion

We confirmed here a key role of the regulatory mechanism of inhibiting JNK activity by means of binding of GST P1-1 to JNK using GST P1-1 mutants of the C-terminal region and the active center domain. GSH-DXR induced JNK activation following the induction of apoptosis via the mitochondrial pathway. Although binding of the C-terminal region of GST P1-1 to the JNK molecule inhibited JNK activity, allosteric inhibition of GST P1-1 avtivity by binding of GSH-DXR following conformational change in the active center region enhanced JNK activity following the induction of apoptosis.

References

1. David RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103:239–252
2. Ichijo H (1999) From receptors to stress-activated MAP kinases. Oncogene 18:6087–6093
3. Ip YT, Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) from inflammation to development. Curr Opin Cell Biol 10:205–219
4. Weston CR, Davis RJ (2002) The JNK signal transduction pathway. Curr Opin Genet Dev 12:14–21
5. Yin Z, Ivanov VN, Habelhah H, Tew KD, Ronai Z (2000) Glutathione S-transferase *p* elicits protection against H_2O_2-induced cell death via coordinated regulation of stress kinases. Cancer Res 60:4053–4057
6. Adler V, Yin Z, Fuchs SY, et al (1999) Regulation of JNK signaling by GSTp. EMBO J 18:1321–1334
7. Wang T, Arifoglu P, Ronai Z, Tew KD (2001) Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the *c* terminus. J Biol Chem 276:20999–21003
8. Adler V, Pincus MR (2004) Effector peptides from glutathione-S-transferase-pi affect the activation of jun by jun-N-terminal kinase. Ann Clin Lab Sci 34:35–46
9. Chie L, Adler V, Friedman FK, Chung D, Pincus MR (2004) An effector peptide from glutathione-S-transferase-pi strongly and selectively blocks mitotic signaling by oncogenic ras-p21. Protein J 23:235–238
10. Chen YR, Wang X, Templeton D, Davis RJ, Tan TH (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet *c* and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J Biol Chem 271:31929–31936
11. Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR (1998) Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-α in rat mesangial cells. J Biol Chem 273:4027–4034
12. Javelaud D, Besançon F (2001) NF-κB activation results in rapid inactivation of JNK in TNFα-treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-κB. Oncogene 20:4365–4372
13. Sanchez-Perez I, Murguia JR, Perona R (1998) Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 16:533–540
14. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331
15. Park H-S, Lee J-S, Huh S-H, Seo J-S, Choi E-J (2001) Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. EMBO J 20:446–456
16. Shim J, Lee H, Park J, Kim H, Choi E-J (1996) A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. Nature 381:804–806
17. Shim J, Park H-S, Kim M-J et al (2000) Rb protein down-regulates the stress-activated signals through inhibiting c-Jun N-terminal kinase/stress-activated protein kinase. J Biol Chem 275:14107–14111
18. Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 281:1671–1674
19. Wilce MCJ, Parker MW (1994) Structure and function of glutathione S-transferases. Biochim Biophys Acta 1205:1–18
20. Nishihira J, Ishibashi T, Sakai M, Nishi S, Kumazaki T (1992) Evidence for the involvement of tryptophan 38 in the activity site of glutathione S-transferase P. Biochem Biophys Res Commun 185:1069–1077
21. Nishihira J, Ishibashi T, Sakai M et al (1992) Characterization of cysteine residues of glutathione S-transferase P: evidence for steric hindrance of substrate binding by a bulky adduct to cysteine 47. Biochem Biophys Res Commun 188:424–432
22. Takahashi N, Asakura T, Ohkawa K (1996) Pharmacokinetic analysis of protein-conjugated doxorubicin (DXR) and its degraded adducts in DXR-sensitive and -resistant rat hepatoma cells. Anti-Cancer Drugs 7:687–696
23. Asakura T, Takahashi N, Takada K, Inoue T, Ohkawa K (1997) Drug conjugate of doxorubicin with glutathione is a potent reverser of multidrug resistance in rat hepatoma cells. Anti-Cancer Drugs 8:199–203
24. Asakura T, Sawai T, Hashidume Y, Ohkawa Y, Yokoyama S, Ohkawa K (1999) Caspase-3 activation during doxorubicin conjugated with glutathione-mediated apoptosis. Br J Cancer 80:711–715
25. Asakura T, Ohkawa K, Takahashi N, Takada K, Inoue T, Yokoyama S (1997) Glutathione-doxorubicin conjugate expresses potent cytotoxicity by a suppression of glutathione S-transferase activity: comparison between doxorubicin-sensitive and -resistant rat hepatoma cells. Br J Cancer 76:1333–1337
26. Asakura T, Hashizume Y, Tashiro K et al (2001) Suppression of GST-P by treatment with glutathione-doxorubicin conjugate induces potent apoptosis in rat hepatoma cells. Int J Cancer 94:171–177
27. Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell 85:803–815

28. Muzio M, Chinnaiyan AM, Kischkel FC et al (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85:817–827
29. Reed JC (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 34:9–19
30. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri ES (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/CE-like cysteine proteases. Proc Natl Acad Sci USA 93:14486–14491
31. Shiraishi H, Okamoto H, Yoshimura A, Yoshida H (2006) ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. J Cell Sci 119:3958–3966
32. Gao G, Dou QP (2000) N-terminal cleavage of Bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes Bcl-2-independent cytochrome *c* release and apoptotic cell death. J Cell Biochem 80:53–72
33. Ghibelli L, Coppola S, Fanelli C et al (1999) Glutathione depletion causes cytochrome *c* release even in the absence of cell commitment to apoptosis. FASEB J 13:2031–2036
34. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1:949–957
35. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405–413
36. Zou H, Li Y, Liu X, Wang X (1999) An APAF-1 cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 274:11549–11556
37. Sugioka Y, Kano T, Okuda A, Sakai M, Kitagawa T, Muramatsu M (1985) Cloning and the nucleotide sequence of rat glutathione S-transferase P cDNA. Nucleic Acids Res 13:6049–6057
38. Ohkawa K, Hatano T, Tsukada Y, Matsuda M (1993) Chemotherapeutic efficacy of the protein-doxorubicin conjugates on multidrug resistant rat hepatoma cell line *in vitro*. Br J Cancer 67:274–278
39. Ohkawa K, Hatano T, Yamada K et al (1993). Bovine serum albumin-doxorubicin conjugate overcomes multidrug resistance in a rat hepatoma. Cancer Res 53:4238–4242
40. Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases—The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139
41. Nicholson DW, Ali A, Thornberry NA et al (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43
42. Hashizume Y, Asakura T, Yamauchi T, Soda K, Ohkawa K (2001) Relationship between cytocidal activity and glutathione S-transferase inhibition using doxorubicin coupled to stereoisomers of glutathione which are different substrate specificity. Anti-Cancer Drugs 12:549–554
43. Fuchs SY, Adler V, Pincus MR, Ronai Z (1998) MEKK1/JNK stabilizes and activates p53. Proc Natl Acad Sci USA 95:10541–10546
44. Kasibhatla S, Brunner T, Genestier L et al (1998) DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol Cell 1:543–551
45. Kobayashi K, Tsukamoto I (2001) Prolonged Jun N-terminal kinase (JNK) activation and the upregulation of p53 and p21 (WAF1/CIP1) preceded apoptosis in hepatocytes after partial hepatectomy and cisplatin. Biochim Biophys Acta 1537:79–88
46. Freeman BC, Myers MP, Schumacher R, Morimoto RI (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J 14:2281–2292
47. Milarski KL, Morimoto RI (1989) Mutational analysis of the human HSP70 protein: distinct domains for nucleolar localization and adenosine triphosphate binding. J Cell Biol 109:1947–1962
48. Li YS, Shyy JY, Li S et al (1996) The Ras-JNK pathway is involved in shear-induced gene expression. Mol Cell Biol 16:5947–5954

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 366 (2008) 471–475
BBRC
www.elsevier.com/locate/ybbrc

FGF-2 signaling induces downregulation of TAZ protein in osteoblastic MC3T3-E1 cells

Homare Eda [a,b], Katsuhiko Aoki [a], Keishi Marumo [b], Katsuyuki Fujii [b], Kiyoshi Ohkawa [a,*]

[a] *Department of Biochemistry, The Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan*
[b] *Department of Orthopaedic Surgery, The Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan*

Received 22 November 2007
Available online 5 December 2007

Abstract

Transcriptional coactivator with PDZ-binding motif (TAZ) protein is a coactivator of Runx2 and corepressor of PPARγ. It also induces differentiation of mesenchymal cells into osteoblasts. In this study, we found that FGF-2, which inhibits bone mineralization and stimulates cell proliferation, reduced the TAZ protein expression level in osteoblast-like cells, MC3T3-E1. This reduction was recovered by removing FGF-2 from the culture medium, which also restored the osteoblastic features of MC3T3-E1 cells. Furthermore, FGF-2-induced reduction of TAZ is blocked by a SAPK/JNK-specific inhibitor. These findings suggest that the expression of TAZ protein is involved in osteoblast proliferation and differentiation. This may help elucidate the discrepancies in the effect of FGF-2 and contribute to the understanding of FGF/FGFR-associated craniosynostosis syndrome etiology and treatment.

Keywords: TAZ; Fibroblast growth factor-2; Osteoblast differentiation; MAP kinase signaling

Transcriptional coactivator with PDZ-binding motif (TAZ) was first reported as a 14-3-3-binding protein in the cytoplasm [1] that interacts with a variety of transcription factors and exhibits transcriptional regulatory functions. TAZ is believed to regulate gene expression during embryogenesis [2] and development of bone [3,4], muscle [5], fat [4], lung [6], heart, and limb [7]. Furthermore, a recent study indicates that TAZ acts as a transcriptional regulator for the differentiation of mesenchymal stem cells into osteoblast cells [4]. In this case, TAZ functions as a coactivator of Runx2, which is a master regulator of osteoblast differentiation. Simultaneously, it also acts as a corepressor of PPARγ, which is a master regulator of adipocyte differentiation. These reports reveal that TAZ plays an important role in mesenchymal stem cell differentiation; however, the regulatory mechanism of TAZ expression in osteoblasts has not been elucidated.

The proliferative expansion of mesenchymal cells, osteoprogenitor cells, and preosteoblasts in response to mitotic growth factors is critical for skeletal development and bone formation. Bone matrix contains large quantities of growth factors, which modulate bone formation by stimulating osteoblast proliferation and differentiation. Among these growth factors, fibroblast growth factor-2 (FGF-2) plays an important role in the control of osteogenesis during skeletal development [8]. It has been shown that in mice with disrupted FGF-2 gene (loss of function), osteoblast proliferation and differentiation are decreased, resulting in significant reduction in bone mass with ageing [9,10]. In transgenic mice, where FGF-2 is overexpressed (gain of function), reduction of osteoblast differentiation is also observed, resulting in abnormal bone phenotype, such as shortening and flattening of long bones [11,12]. Although FGF-2 is a potential mitogen for osteoblast cell lineage and increased bone formation, continuous treatment with a high concentration of FGF-2 inhibits differentiated function of osteoblasts by suppressing synthesis of type I collagen and other proteins [13–15]. FGF-2 activates several

* Corresponding author. Fax: +81 3 5400 1251.
E-mail address: pko@jikei.ac.jp (K. Ohkawa).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.11.140

signal transduction pathways in osteoblasts, including mitogen-activated protein (MAP) kinase [16,17]; however, the mechanism of the pathway and its effects on osteoblast function have not yet been understood.

In this study, we found that the intracellular level of TAZ protein in osteoblastic MC3T3-E1 cells reduces by treatment with FGF-2, not other osteogenic cytokines, and almost completely disappears after 2 days. This reduction was mediated by a stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) that is downstream of the FGF signal transduction pathway. The intracellular level of TAZ protein is recovered by removing FGF-2 from the culture medium.

Materials and methods

Materials. Human recombinant FGF-basic (FGF-2), BMP-2, BMP-7, and TGF-β1 were purchased from PeproTech EC Ltd. (London, UK); additional BMP-7 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Anti-TAZ antibody was purchased from GeneTex Inc. (San Antonio, TX, USA); anti-GAPDH antibody (clone 6C5) was purchased from Chemicon International Inc. (currently Millipore Corp.); and secondary antibodies (Histofine Simple Stain AP[M] and AP[R]) were purchased from Nichirei Corp. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from MP Biomedicals Inc. (Aurora, OH, USA); and α-Minimum Essential Medium (α-MEM) was purchased from Invitrogen Corp. (Carlsbad, CA, USA). MAP kinase inhibitors (SP600125, SB202190 and PD98059) were purchased from Tocris Cookson Ltd. (Bristol, UK).

Cell cultures. The osteoblast-like cell line MC3T3-E1 was maintained in α-MEM supplemented with 10% FBS [18,19]. To induce osteoblast differentiation, the cells were grown in 100-mm plates at subconfluent state, and then the cells were trypsinized and subcultured at 30,000–50,000 cells/cm^2 in a 6-well plate. After 24 h, the medium was replaced with differentiation medium (α-MEM containing 10% FBS with 2 mM L-ascorbic acid 2-phosphate and 4 mM β-glycerophosphate). The media were replaced every 3 days. All the cells were incubated in a humidified incubator with 5% CO_2 at 37 °C.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). RNA isolation and RT-PCR were carried out according to the manufacturer's specifications. Total cellular RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI, USA) and its concentration was determined using a spectrophotometer. Reverse transcription was carried out using Transcriptor Reverse Transcriptase (Roche Applied Science, Mannheim, Germany) with oligo-dT (15mer) primer. cDNAs were then amplified using GoTaq® Green Master Mix (Promega) with a pair of gene-specific primers. Primer sequences for the analyzed genes were as follows: TAZ: 5′-GTCACCAACA GTAGCTCAGA TC-3′ and 5′-AGTGATTACA GCCAGGTTAG AAAG-3′; osteocalcin: 5′-AC CATGAGGA CCATCTTTCT-3′ and 5′-CTGCTGTGAC ATCCATAC TT-3′; aP2: 5′-TGATGCCTTT GTGGGAACCT-3′ and 5′-TTTGGCTC AT GCCCTTTCAT-3′; GAPDH: 5′-ACTTTGGCAT TGTGGAAGG G-3′ and 5′-TTACTCCTTG GAGGCCATGT-3′.

Immunoblotting. Cells were washed with phosphate-buffered saline (PBS) and harvested. Harvested cells were lysed with SDS–PAGE sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue) and immediately boiled at 100 °C for 5 min. The whole cell extracts were quantified with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and 30 μg of each sample was subjected to SDS–PAGE on 12% acrylamide gel. After SDS–PAGE, proteins were transferred to nitrocellulose membranes. The blotted membranes were blocked with blocking buffer (Tris-bufferd saline, 0.05% Tween 20, 0.1% gelatin, 0.1% casein) and then incubated with primary antibodies (1:1000 dilution) in blocking buffer. After washing the membranes, the bound primary antibodies were detected with alkaline phosphatase-labeled secondary antibodies (1:200 dilution) and visualized by BCIP/NBT color development reaction.

Alizarin red S staining. The mineralization of MC3T3-E1 cells was determined in 6-well plates using Alizarin red S staining. After the confluent cells were grown in differentiation medium for 21 days, the cells were fixed in 10% formalin/PBS for 10 min and stained with 1% Alizarin red S in each well for 30 min. The dye from the wells was washed thoroughly with distilled water and dried.

Results and discussion

FGF-2 regulates intracellular protein level of TAZ

Since it is known that TAZ binds to Runx2 and promotes its transcriptional function on the osteocalcin promoter [3,4], we investigated the regulatory mechanism of TAZ expression in MC3T3-E1 cells. To identify the effect of osteogenic cytokines on the expression of TAZ, we analyzed the TAZ protein level in the MC3T3-E1 cells with immunoblotting (Fig. 1A). Interestingly, TAZ protein was diminished in the MC3T3-E1 cells were treated with FGF-2 (100 ng/ml) for 48 h, but its expression was not affected or was slightly increased in the presence of other osteogenic cytokines, such as BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), BMP-7 (100 ng/ml), and TGF-β1 (50 ng/ml). In unpublished observations, Hong and Yaffe noted that TAZ levels increased several fold when mesenchymal stem cells were stimulated to differentiate into osteoblasts by treatment with BMP-2 [20]. However as shown in Fig. 1A, such upregulation of TAZ protein was not observed. This is probably because of the MC3T3-E1 cells that already exhibit some features of osteoblasts. In addi-

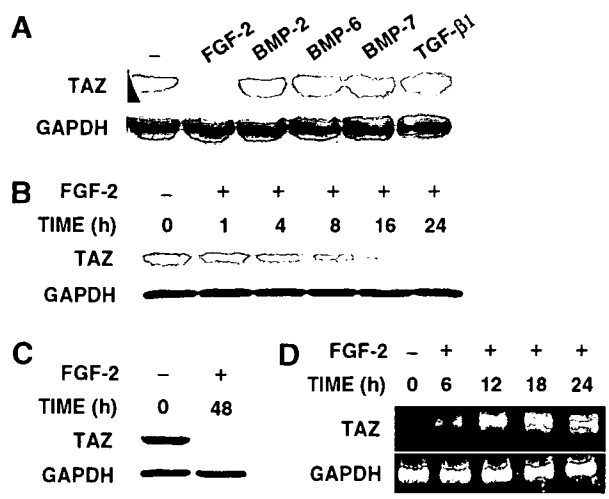

Fig. 1. Effect of osteogenic cytokines on the expression of TAZ protein and mRNA. TAZ and GAPDH proteins were analyzed by immunoblotting of whole cell extract (30 μg protein) (A–C). TAZ and GAPDH mRNA were analyzed by RT-PCR (D). (A) MC3T3-E1 cells were either not treated (–) or treated with FGF-2 (100 ng/ml), BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), BMP-7 (100 ng/ml), and TGF-β1 (50 ng/ml) for 48 h. (B–D) MC3T3-E1 cells were not treated (–) or treated (+) with 100 ng/ml FGF-2 at the given time.

tion, a clear reduction of TAZ protein in a time-dependent manner was observed (Fig. 1B and C). TAZ protein gradually decreased from 4 to 24 h after FGF-2 treatment and almost completely disappeared after 48 h. At these points, surprisingly, mRNA expression of TAZ was gradually upregulated as opposed to protein downregulation (Fig. 1D). Therefore, the reduction of TAZ protein might not regulate a transcriptional but instead a translational or posttranslational process. Our preliminary experiments confirm that TAZ protein reduction was not blocked by protease inhibitors (e.g. serine protease inhibitor, AEBSF; cysteine protease inhibitor, E64d; aspartic protease inhibitor, pepstatin-AM; calcium chelator, EGTA-AM; calpain inhibitor, PD150606; lysosome inhibitor, NH_4Cl and proteasome inhibitor, MG132) (data not shown). Further investigation will be required to elucidate the mechanism underlying the downregulation of TAZ protein by FGF-2 treatment in MC3T3-E1 cells.

FGF-2-induced reduction of TAZ protein was mediated by SAPK/JNK signaling

It is currently known that the MAP kinase mediates intracellular signaling of FGF-2 in osteoblasts and plays a crucial role in cellular functions including proliferation, differentiation, and cell death [16,17]. Three major MAP kinases, SAPK/JNK, p38 MAP kinase (p38) and extracellular signal-regulated kinase-1/2 (ERK1/2) are known as central elements in mammalian cells. To determine which MAP kinase is involved in the reduction of TAZ protein, specific inhibitors to each MAP kinase pathway were utilized as follows: SP600125, characterized previously as a specific inhibitor for SAPK/JNK [21]; SB202190, identified as a p38-specific inhibitor [22] and PD98059, shown to act as a highly selective inhibitor of MEK1, which is the kinase upstream of ERK1/2 [23]. As shown in Fig. 2A, SP600125 inhibited the reduction of TAZ protein in a dose-dependent manner in the presence of FGF-2. In contrast, SB202190 and PD98059 did not affect the reduction of TAZ protein (Fig. 2B and C). These results clearly indicate that the reduction of TAZ protein is mediated by SAPK/JNK signaling, which is downstream of the FGF signal transduction pathway. Earlier studies indicated that FGF-2 has a proliferative effect on rat and human primary osteoblasts as well as mouse osteoblastic MC3T3-E1 cells [8]. Certainly, incorporation of BrdU, which is used to observe DNA replication in MC3T3-E1 cells is enhanced by FGF-2 treatment (data not shown). Furthermore, MC3T3-E1 cells changed from fibroblast-shaped to spindle-shaped cells after treatment with FGF-2 (data not shown). Since TAZ is assumed to be an important factor in the differentiation of mesenchymal stem cells into osteoblasts [4], the diminution of TAZ protein caused by treatment with FGF-2 possibly gave rise to the shift from osteoblastic differentiation state to proliferation state of MC3T3-E1 cells.

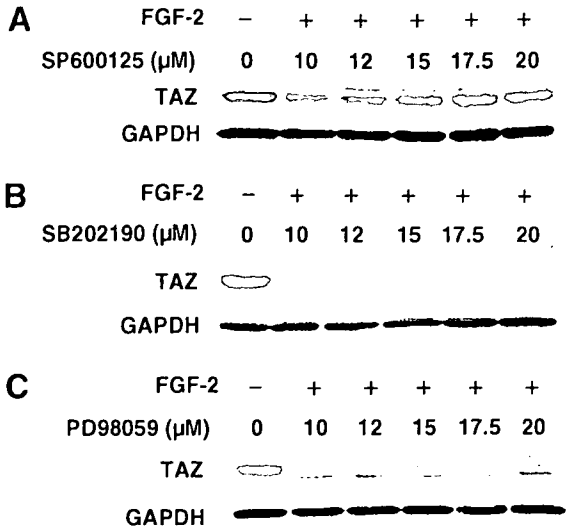

Fig. 2. Involvement of MAP kinase on the reduction of TAZ protein. MC3T3-E1 cells were treated with predetermined concentrations of MAP kinase inhibitors specific for SAPK/JNK (SP600125) (A), p38 (SB202190) (B), and MEK1, which is the kinase upstream of ERK1/2 (PD98059) (C), respectively. After the addition of these inhibitors, the cells were either not treated (–) or treated (+) with 100 ng/ml FGF-2 for 48 h. TAZ and GAPDH proteins were analyzed by immunoblotting of the whole cell extract (30 μg protein).

Osteoblastic feature of MC3T3-E1 cells was recovered by removing FGF-2 from the culture medium simultaneous increase of TAZ protein

Recently, it was reported that TAZ protein functions as a coactivator of Runx2, which is a master regulator of osteoblast differentiation in mesenchymal stem cells. Simultaneously it functions as a corepressor of PPARγ, which is a master regulator of adipocyte differentiation. Here, we demonstrated the downregulation of TAZ protein by treatment with FGF-2 of the osteoblastic MC3T3-E1 cells. Thus, in our study, the expression of osteocalcin mRNA, which is targeted by Runx2, should be downregulated. Conversely, the expression of aP2 mRNA, which is targeted by PPARγ may be upregulated. The RT-PCR analysis confirmed that the osteocalcin mRNA gradually decreased after treatment with FGF-2, whereas the aP2 mRNA increased (Fig. 3A). In addition, with the removal of FGF-2 from the culture medium the osteocalcin mRNA expression was shifted from a reduced to an elevated state (Fig. 3B). Conversely, the aP2 mRNA expression was shifted from an elevated to a reduced state after substitution of the medium. Furthermore, we analyzed the alteration in the expression level of TAZ protein after substitution of FGF-2-containing culture medium with FGF-2-free medium (Fig. 3C). The results of immunoblotting indicate that the content of TAZ protein could be gradual increased from the reduced condition by the treatment with FGF-2. It is known that continuous treatment with FGF-2 inhibits the expression of preosteoblastic

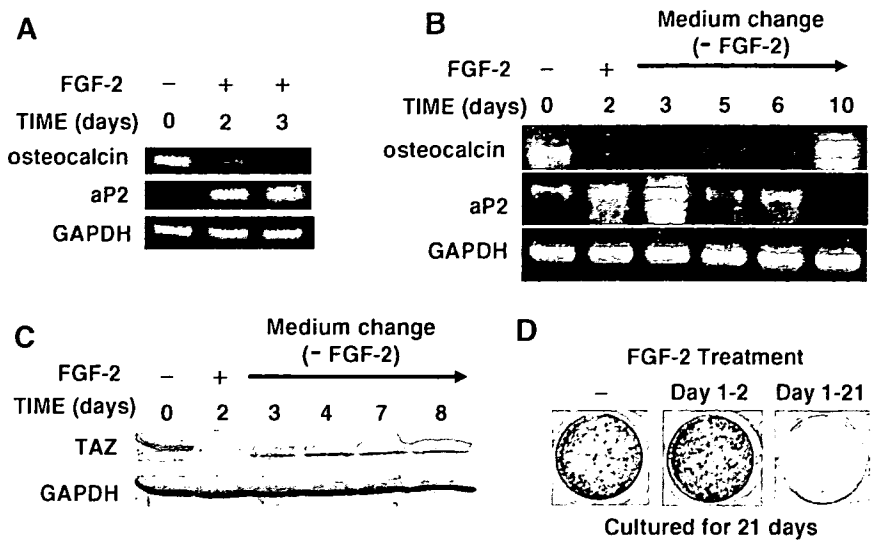

Fig. 3. Recovery of osteogenic features in MC3T3-E1. Osteocalcin, aP2 and GAPDH mRNA were analyzed by RT-PCR. MC3T3-E1 cells were either not treated (−) or treated (+) with 100 ng/ml FGF-2 for the given time (A,B). (B) After the treatment with FGF-2 for 2 days, the culture medium was replaced with FGF-2-free medium and the cells were cultured for various time periods (up to 10 days). (C) TAZ and GAPDH proteins were analyzed by immunoblotting of whole cell extract (30 μg protein). MC3T3-E1 cells were either not treated (−) or treated (+) with 100 ng/ml FGF-2 for the given time. After the treatment with FGF-2 for 2 days, the culture medium was replaced with FGF-2-free medium and the cells were cultured for various time periods (up to 8 days). (D) The mineralization of MC3T3-E1 cells was determined using Alizarin red S staining. MC3T3-E1 cells were either not treated (−) or treated with 100 ng/ml FGF-2 transiently (Day 1–2) or continuously (Day 1–21).

markers (alkaline phosphatase and type I collagen) and mature osteoblastic markers (osteocalcin and bone syaloprotein) in murine marrow stromal cells and human calvaria osteoblastic cells. Further, in long-term culture, continuous treatment with FGF-2 also inhibited mineralization, which caused osteoblastic differentiation of these cells and MC3T3-E1 cells [15,24,25]. In Fig. 3D, we confirmed the inhibition of mineralization by continuous treatment with FGF-2 (Day 1–21) in MC3T3-E1. By contrast, the inhibition of mineralization was not found by transient 2 days treatment with FGF-2 (Day 1–2). The transient period of FGF-2 exposure is sufficient to abolish the TAZ protein expression in MC3T3-E1 cells. And then, after the proliferation, osteoblastic features may be recovered concomitant with the recovery of TAZ protein expression. Taken together, these results suggest that differentiating ability with osteoblastic feature of MC3T3-E1 cells was restored from proliferation state, which was produced by the treatment of FGF-2.

This study is the first to reveal that FGF-2 reduces the endogenous TAZ protein level mediated by the SAPK/JNK signaling pathway, and this reduction was recovered by removing FGF-2 from the culture medium. Previous studies have indicated that FGF-2 has both stimulatory and inhibitory effects on bone formation *in vitro* and *in vivo*. It was reported that FGF-2 inhibits extracellular matrix protein synthesis and mineralization, which is caused by osteoblastic differentiation *in vitro* [13,14,25]. FGF-2 transgenic mice (overexpression and knockout) displayed abnormal bone phenotypes resulting in a decrease in osteoblast differentiation [9,11,12]. Furthermore, persistent FGF signaling by mutations in FGF receptor (FGFR) were responsible for several clinically distinct craniosynostosis syndromes in humans [26]. In contrast, it was reported that FGF-2 increases osteoblast differentiation and extracellular matrix mineralization *in vitro* [27–32]. In addition, FGF-2 accelerates fracture healing and bone formation *in vivo* [33–38]. As a result, little is known about causes of discrepancies in the effects of FGF-2. In our study, TAZ expression was regulated by FGF-2, suggesting that the expression of TAZ protein is involved in osteoblast proliferation and differentiation. This may help elucidate the discrepancies and contribute to the understanding of FGF/FGFR-associated craniosynostosis syndrome etiology and treatment.

Acknowledgments

The authors express their gratitude to Drs. Koji Takada and Mitsuru Saito for their helpful suggestions and critique of the manuscript. This research was supported by The Jikei University Research Fund.

References

[1] F. Kanai, P.A. Marignani, D. Sarbassova, R. Yagi, R.A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito, L.C. Cantley, M.B. Yaffe, TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins, EMBO J. 19 (2000) 6778–6791.

[2] M. Murakami, J. Tominaga, R. Makita, Y. Uchijima, Y. Kurihara, O. Nakagawa, T. Asano, H. Kurihara, Transcriptional activity of Pax3 is co-activated by TAZ, Biochem. Biophys. Res. Commun. 339 (2006) 533–539.

[3] C.B. Cui, L.F. Cooper, X. Yang, G. Karsenty, I. Aukhil, Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ, Mol. Cell. Biol. 23 (2003) 1004–1013.
[4] J.H. Hong, E.S. Hwang, M.T. McManus, A. Amsterdam, Y. Tian, R. Kalmukova, E. Mueller, T. Benjamin, B.M. Spiegelman, P.A. Sharp, N. Hopkins, M.B. Yaffe, TAZ, a transcriptional modulator of mesenchymal stem cell differentiation, Science 309 (2005) 1074–1078.
[5] W.M. Mahoney Jr, J.H. Hong, M.B. Yaffe, I.K.G. Farrance, The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members, Biochem. J. 388 (2005) 217–225.
[6] K.S. Park, J.A. Whitsett, T.D. Palma, J.H. Hong, M.B. Yaffe, M. Zannini, TAZ interacts with TTF-1 and regulates expression of surfactant protein-C, J. Biol. Chem. 279 (2004) 17384–17390.
[7] M. Murakami, M. Nakagawa, E.N. Olson, O. Nakagawa, A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome, Proc. Natl. Acad. Sci. USA 102 (2005) 18034–18039.
[8] M.M. Hurley, P.J. Marie, R.Z. Florkiewicz, Fibroblast growth factor (FGF) and FGF receptor families in bone, Principles of Bone Biology, Academic Press, New York, 2002, pp. 825–851.
[9] A. Montero, Y. Okada, M. Tomita, M. Ito, H. Tsurukami, T. Nakamura, T. Doetschman, J.D. Coffin, M.M. Hurley, Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation, J. Clin. Invest. 105 (2000) 1085–1093.
[10] T. Naganawa, L. Xiao, E. Abogunde, T. Sobue, I. Kalajzic, M. Sabbieti, D. Agas, M.M. Hurley, In vivo and in vitro comparison of the effects of FGF-2 null and haplo-insufficiency on bone formation in mice, Biochem. Biophys. Res. Commun. 339 (2006) 490–498.
[11] J.D. Coffin, R.Z. Florkiewicz, J. Neumann, T. Mort-Hopkins, G.W. Dorn II, P. Lightfoot, R. German, P.N. Howles, A. Kier, B.A. O'Toole, J. Sasse, A.M. Gonzalez, A. Baird, T. Doetschman, Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice, Mol. Biol. Cell 6 (1995) 1861–1873.
[12] T. Sobue, T. Naganawa, L. Xiao, Y. Okada, Y. Tanaka, M. Ito, N. Okimoto, T. Nakamura, J.D. Coffin, M.M. Hurley, Over-expression of fibroblast growth factor-2 causes defective bone mineralization and osteopenia in transgenic mice, J. Cell. Biochem. 95 (2005) 83–94.
[13] E. Canalis, M. Centrella, T. McCarthy, Effects of basic fibroblast growth factor on bone formation in vitro, J. Clin. Invest. 81 (1988) 1572–1577.
[14] M.M. Hurley, C. Abreu, J.R. Harrison, A.C. Lichtler, L.G. Raisz, B.E. Kream, Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells, J. Biol. Chem. 268 (1993) 5588–5593.
[15] F. Debiais, M. Hott, A.M. Graulet, P.J. Marie, The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific, J. Bone Miner. Res. 13 (1998) 645–654.
[16] P.J. Marie, Fibroblast growth factor signaling controlling osteoblast differentiation, Gene 316 (2003) 23–32.
[17] C. Ge, G. Xiao, D. Jiang, R.T. Franceschi, Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development, J. Cell Biol. 176 (2007) 709–718.
[18] H. Sudo, H.A. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, J. Cell Biol. 96 (1983) 191–198.
[19] S.I. Harada, T. Matsumoto, E. Ogata, Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells, J. Bone Miner. Res. 6 (1991) 903–908.
[20] J.H. Hong, M.B. Yaffe, TAZ: a β-catenin-like molecule that regulates mesenchymal stem cell differentiation, Cell cycle 5 (2006) 176–179.
[21] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, Proc. Natl. Acad. Sci. USA 98 (2001) 13681–13686.
[22] S. Nemoto, J. Xiang, S. Huang, A. Lin, Induction of Apoptosis by SB202190 through inhibition of p38β mitogen-activated protein kinase, J. Biol. Chem. 273 (1998) 16415–16420.
[23] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo, J. Biol. Chem. 270 (1995) 27489–27494.
[24] I. Kalajzic, Z. Kalajzic, M.M. Hurley, A.C. Lichtler, D.W. Rowe, Stage specific inhibition of osteoblast lineage differentiation by FGF2 and noggin, J. Cell. Biochem. 88 (2003) 1168–1176.
[25] N.E. Hatch, F. Nociti, E. Swanson, M. Bothwell, M. Somerman, FGF2 alters expression of the pyrophosphate/phosphate regulating proteins, PC-1, ANK and TNAP, in the calvarial osteoblastic cell line, MC3T3E1(C4), Connect. Tissue Res. 46 (2005) 184–192.
[26] D.M. Ornitz, P.J. Marie, FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease, Genes Dev. 16 (2002) 1446–1465.
[27] D. Noff, S. Pitaru, N. Savion, Basic fibroblast growth factor enhances the capacity of bone marrow cells to form bone-like nodules in vitro, FEBS Lett. 250 (1989) 619–621.
[28] S. Pitaru, S. Kotev-Emeth, D. Noff, S. Kaffuler, N. Savion, Effect of basic fibroblast growth factor on the growth and differentiation of adult stromal bone marrow cells: enhanced development of mineralized bone-like tissue in culture, J. Bone Miner. Res. 8 (1993) 919–929.
[29] I. Martin, A. Muraglia, G. Campanile, R. Cancedda, R. Quarto, Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow, Endocrinology 138 (1997) 4456–4462.
[30] S. Pri-Chen, S. Pitaru, F. Lokiec, N. Savion, Basic fibroblast growth factor enhances the growth and expression of the osteogenic phenotype of dexamethasone-treated human bone marrow-derived bone-like cells in culture, Bone 23 (1998) 111–117.
[31] A. Scutt, P. Bertram, Basic fibroblast growth factor in the presence of dexamethasone stimulates colony formation, expansion, and osteoblastic differentiation by rat bone marrow stromal cells, Calcif. Tissue Int. 64 (1999) 69–77.
[32] S. Walsh, C. Jefferiss, K. Stewart, G.R. Jordan, J. Screen, J.N. Beresford, Expression of the developmental markers STRO-1 and alkaline phosphatase in cultures of human marrow stromal cells: regulation by fibroblast growth factor (FGF)-2 and relationship to the expression of FGF receptors 1–4, Bone 27 (2000) 185–195.
[33] J.S. Wang, Basic fibroblast growth factor for stimulation of bone formation in osteoinductive or conductive implants, Acta Orthop. Scand. Suppl. 269 (1996) 1–33.
[34] T. Kato, H. Kawaguchi, K. Hanada, I. Aoyama, Y. Hiyama, T. Nakamura, K. Kuzutani, M. Tamura, T. Kurokawa, K. Nakamura, Single local injection of recombinant fibroblast growth factor-2 stimulates healing of segmental bone defects in rabbits, J. Orthop. Res. 16 (1998) 654–659.
[35] T. Nakamura, Y. Hara, M. Tagawa, M. Tamura, T. Yuge, H. Fukuda, H. Nigi, Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture, J. Bone Miner. Res. 13 (1998) 942–949.
[36] M.L. Radomsky, A.Y. Thompson, R.C. Spiro, J.W. Poser, Potential role of fibroblast growth factor in enhancement of fracture healing, Clin. Orthop. Relat. Res. 355S (1998) S283–S293.
[37] G. Zellin, A. Linde, Effects of recombinant human fibroblast growth factor-2 on osteogenic cell populations during orthopic osteogenesis in vivo, Bone 26 (2000) 161–168.
[38] H. Kawaguchi, K. Nakamura, Y. Tabata, Y. Ikada, I. Aoyama, J. Anzai, T. Nakamura, Y. Hiyama, M. Tamura, Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2, J. Clin. Endocrinol. Metab. 86 (2001) 875–880.

Online Submissions: wjg.wjgnet.com
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 October 28; 13(40): 5306-5311
World Journal of Gastroenterology ISSN 1007-9327

LIVER CANCER

Survivin expression in early hepatocellular carcinoma and post-treatment with anti-cancer drug under hypoxic culture condition

Satoshi Mamori, Tadashi Asakura, Kiyoshi Ohkawa, Hisao Tagiri

Satoshi Mamori, Hisao Tajiri, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Tadashi Asakura, Kiyoshi Ohkawa, Department of Biochemistry 1, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Correspondence to: Satoshi Mamori, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. mamori@jikei.ac.jp
Telephone: +81-3-34331111-3201 Fax: +81-3-34350569
Received: July 07, 2007 Revised: August 17, 2007

Abstract

AIM: To investigate the expression of survivin during the early stages of hepatocellular carcinoma (HCC).

METHODS: Immunohistochemical expression of survivin in liver tumor and non-tumor tissue specimens taken from 17 patients was compared. In addition, to determine the survivin expression in response to anti-cancer drugs in early stage HCC, the survivin expression was determined after the treatment of the HCC cells with anti-cancer drugs under hypoxic culture conditions.

RESULTS Survivin proteins were expressed in 64.7% of cells in early HCC specimens. A correlation between the survivin expression rate in the peritumoral hepatocytes and the rate of expression in the HCC specimens (low-rate groupvs high-rate group) was observed. The survivin protein concentration in HCC cells was increased by the combination of hypoxia and anti-cancer drugs.

CONCLUSION: This study suggests that survivin could be used as a therapeutic target in early HCC.

Key words: Survivin; Hepatocellular carcinoma; Hypoxia

Mamori S, Asakura T, Ohkawa K, Tagiri H. Survivin expression in early hepatocellular carcinoma and post-treatment with anti-cancer drug under hypoxic culture condition. *World J Gastroenterol* 2007; 13(40): 5306-5311

http://www.wjgnet.com/1007-9327/13/5306.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide. There are more than 500 000 new cases diagnosed each year, with an age-adjusted incidence of 5.5-14.9 per 100 000 people[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death[2]. The incidence of HCC is also increasing in Europe and the United States[3]. A more effective therapy thus needs to be developed from early stages.

Survivin is a member of a family of inhibitors of apoptosis protein (IAP), which has been implicated in both the control of cell division and the inhibition of apoptosis. Specifically, its anti-apoptotic function is associated with the ability to directly or indirectly inhibit caspases. By inhibiting apoptosis and promoting mitosis, survivin facilitates cancer cell survival and growth[4-8]. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation[9].

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function by the use of anti-sense oligonucleotide, dominant negative mutants, ribozymes, small interfering RNAs and cyclin-dependent kinase inhibitors increased the rate of apoptosis, reduced tumor growth potential and sensitized tumor cells to various chemotherapeutic drugs and γ-irradiation in *in vitro* and *in vivo* models of various types of human tumors[9]. Moreover, YM155 is the first agent designed to inhibit survivin. Some early phase clinical studies demonstrated that this novel anticancer agent was well tolerated and shrank tumors in some patients with non-Hodgkin lymphoma and hormone-refractory prostate cancer that recurred after conventional chemotherapy. In addition, interim reports indicate that there are few side effects.

These results suggest the possible efficacy of the survivin inhibitor on HCC. It may be effective for patients with early stages of HCC. Survivin is expressed in HCC[10]. However, the expression during the early stages of HCC has not been characterized pathologically. In addition, previous results have shown that survivin gene transcription is increased in hypoxic tumor cells[11]. The well-differentiated HCC has portal blood flow and is not hypervascular[12]. In order to compare the expression of

survivin and the efficacy of anti-cancer drugs, HCC cells were cultured in a hypoxic environment.

MATERIALS AND METHODS

Patients

The study population included 17 patients (11 men and 6 women; median age 68 years, range 56-81 years) who underwent a tumor biopsy between January 2004 and December 2005 in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm the diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Committee Institutional Review Broad.

Table 1 Characteristics of the patients undergoing tumor biopsies (*n* = 17)

Features	Values
Age, yr	68 (56-81)
Sex (Male/Female)	11/6
AFP (ng/mL)	21 (3-444)
HBsAg/HCVAb	3/14
Tumor size (mm)	15 (8-23)
Cirrhosis (positive/negative)	5/12
Differentiation (well/moderate)	11/6

Data are expressed as the medians with ranges in parentheses unless indicated otherwise. HBsAg: Anti-hepatitis B surface antigen; HCVAb: Anti-hepatitis C antibody; Well: Well-differentiated HCC; Moderate: Moderate-differentiated HCC; Normal ranges: AFP (alpha-feto protein) > 20 ng/mL.

Pathologic specimens

Tumor specimens were obtained by tumor biopsies with a 21-G fine-needle aspiration kit. Non-tumorous liver tissue specimens were concurrently obtained by an 18-20-G needle liver biopsy. Formalin-fixed, paraffin-embedded specimens of liver tumor and non-tumor tissues were processed for conventional histological assessment by hematoxylin and eosin (H&E) staining. The tumors were histologically graded as well or moderately differentiated.

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were used after deparaffinization. A rabbit anti-human survivin polyclonal antibody (Diagnostic BioSystems, USA) was used at dilution of 1:2000 as the primary antibody, which was detected with ENVISION + Rabbit/HRP (Dako, Japan). The specimens were heated in a microwave oven containing antigen retrieval solution (10 mmol/L citrate buffer, pH 6.4) at 121℃ for 15 min for the retrieval of the antigens and then cooled to room temperature. 3, 3-Diaminobenzidine and hematoxylin were used for color development and counterstaining, respectively. Cells with brown-colored nuclei were regarded as positive. The mean percentage of survivin-positive HCC cells was determined in three areas at 100 × magnification with the nuclear labeling index (labeled nuclei/500 nuclei). The same method was performed for hepatocytes in non-tumorous biopsy specimens.

HCC cell line cultured in the combination of hypoxia and anti-cancer drugs environment

Human hepatocellular carcinoma cell line FLC-7 was cultured with RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS) under conventional conditions at 37℃ in a humidified atmosphere containing 50 mL/L CO_2[13] until the cells were 70%-80% confluent. Cells were then used for culture under hypoxic conditions employing the AnaeroPack for cell (Mitsubishi Gas Chemical Co., Tokyo, Japan) packaging device. The cells were sealed tightly and incubated at 37℃ for either 6 or 96 h. In addition, for the anti-cancer drug therapy, the cells were cultured continuously with 0.1 μmol/L farmorubicin (EPI) containing growth medium for 6 or 96 h. The cytotoxicity (IC50) with EPI of FLC-7 cells determined the medication concentration (Normoxia cultured for 96 h, data not shown).

Table 2 PCR primer sequences

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Survivin	GCCCAGTGTTTCTTCT GCTT	GCACTTTCTTCGCAGT TTCC
β-actin	AGCCATGTACGTAGC CATCC	AAGTGGTGGTGTCGAC TCTC

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the RNeasy kit (Qiagen, Hilden, Germany). The mRNA was reverse transcribed into cDNA using the Prime script (TAKARA BIO INC, Shiga, Japan). The specific cDNA target sequences for survivin were amplified by a PCR reaction mixture consisting of 1 μL cDNA template, 10 μmol/L each primer (Primer sequences are listed in Table 2), PCR Master Mix (Go taq, Promega, Madison, WI, USA). The PCR conditions were: initial pre-denaturation at 95℃ for 5 min; 30 amplification cycles of denaturation at 95℃ for 30 s, primer annealing at 55℃ for 30 s, and elongation at 72℃ for 60 s; and a final extension at 72℃ for 5 min. PCR products were analyzed on a 20 g/L agarose/TBE gel electrophoresis and compared to the expression of β-actin as a housekeeping gene.

Western blotting

The harvested cells cultured in either a normal or hypoxic environment for 96 h with or without 0.1 μmol/L of EPI were washed with ice-cold PBS and lysed in ice-cold 5 mL/L Triton X-100 containing 10 mmol/L EDTA. The cell lysate was centrifuged at 15000 g for 5 min and the supernatant was used for Western blotting. Thirty micrograms of protein was separated on 150 g/L polyacrylamide gels and transferred onto 0.2-μm nitrocellulose membranes by wet blotting (20 mA for 60 min). Membranes were blocked with blocking buffer (1 × TBS, 1 g/L Tween-20, 1 g/L casein gelatin) for 0.5 h at 37℃ and stained with the specific antibody for survivin

Figure 1 Immunopathological staining of survivin in HCC and peritumoral biopsy tissues. A: Non-tumor biopsy (x 100); B: Tumor biopsy (x 100); C: Non-tumor biopsy (x 400); D: Tumor biopsy (x 400).

(1:1000; Novus Biologicals, Littleton, USA). The complex of antigen with the primary antibody was completely labeled with the secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate (1:2000; Sigma-Aldrich Japan, Tokyo, Japan). The survivin band was visualized with 5-bromo-4-chlor-3-indoly-phosphate/nitro blue terazulium (Sigma-Aldrich Japan, Tokyo, Japan).

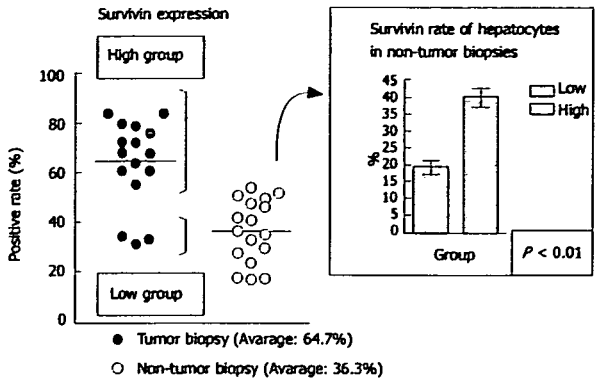

Figure 2 Nuclear survivin expression rates in HCC biopsies and non-tumor biopsies samples. In tumor biopsies, > 500 survivin-expressing HCC cells were counted in three areas at 100 x magnification using the nuclear labeling index. In non-tumor biopsies, > 500 survivin-expressing hepatocyte cell were counted in three areas at 100 x magnification using the nuclear labeling index.

Protein determination

Protein concentration was assayed by a Bio-Rad protein assay kit (Bio-Rad Lab., Tokyo, Japan) using BSA as the standard.

Statistical analysis

Statistical analyses were performed using the Wilcoxon-Mann-Whitney two-sample rank-sum test. A P value less than 0.05 was considered statistically significant.

RESULTS

Rates of survivin expression in early HCC and in non-tumorous liver tissues

In HCC tissues, the rate of survivin expression was determined by counting survivin-positive cancer cells (Figure 1). The average survivin expression rate was 64.7% (median). The samples with a survivin expression rate over 50% were regarded as high-rate group, while the three samples with a survivin expression rate under 50% were regarded as low-rate group (Figure 2). In early-stage HCC with a tumor size > 10 mm (n = 3), the expression rate ranged for 67.7%-83.7%. The expression rate of survivin in HCC had no significant correlation to the level of differentiation of HCC. In non-tumorous liver tissues, survivin expression rates were counted in all hepatocytes. The average of survivin expression was 36.3% (median, Figure 2). A correlation between the survivin expression rate in peritumoral cells and the rate of expression in the HCC specimens (low-rate group *vs* high-rate group) was observed. A significant difference in survivin expression in the hepatocyte was observed between the high-rate group and low-rate group ($P < 0.01$, Figure 2).

Survivin expression of HCC cells in hypoxic conditions and post-treatment with anti-cancer drugs

The hypoxic environment increased the survivin mRNA expression in both short-term and long-term cultures (Figure 3). Under both normoxia and hypoxia, the survivin mRNA concentrations increased in the presence of anti-cancer drugs in the short-term culture.

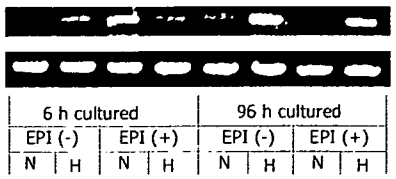

Figure 3 Expression of survivin mRNA in a hypoxic and anti-cancer drug-containing medium. The survivin mRNA expression increased under hypoxia, by anti-cancer drug treatment, and in presence of the both conditions in the 6-h culture. The survivin mRNA expression increased under hypoxia and in the combined conditions of hypoxia and anti-cancer drug in the 96-h culture.

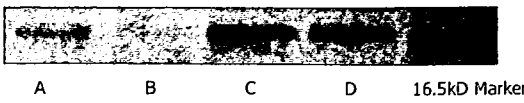

Figure 4 Western blotting showing the expression of survivin protein in the combined conditions of hypoxia and anti-cancer drugs in the 96-h culture. Survivin expression increased under anti-cancer drug-containing medium. Moreover, survivin further increased after the administration of a combination of hypoxia and anti-cancer drug. **A**: Normoxia; **B**: Hypoxia; **C**: Normoxia + 0.1 μmol/L EPI; **D**: Hypoxia + 0.1 μmol/L EPI.

Nevertheless, the survivin mRNA concentrations only increased in the combination of hypoxic culture and anti-cancer drugs in the long-term culture (Figure 3). No survivin protein expression was observed in the hypoxia culture (Figure 4). In contrast, the survivin protein concentration increased with the anti-cancer drug concentrations. Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs (Figure 4).

DISCUSSION

The suppression of apoptosis is thought to contribute to carcinogenesis due to several mechanisms, including unusually prolonging the cellular lifespan, facilitating the accumulation of gene mutations and permitting growth factor-independent cell survival[14]. In addition, since the host's immune system normally eliminates cancer cells by induction of apoptosis, inhibition of this process is critical for cancer cells survival. Several proteins, including the bcl-2 family and the IAP family, are involved in the inhibition of apoptotic signaling[15-16]. Survivin, a novel member of the IAP family, inhibits the activation of caspase-3 and -7, which are downstream effectors of apoptosis, in cells exposed to apoptotic stimuli[17-20]. Previous studies have shown that survivin is expressed at a high level in 60%-100% of the most common human tumor types, including colon, pancreas, breast, lung, liver, brain, lymphoma, melanoma and prostate cancers[21-24]. The elevated expression of survivin is associated with poor patient survival[25-28]. In the present study, positive nuclear survivin expression was observed in all tumor biopsy samples. It is possible that this result was based on dyeing conditions and the nuclear labeling index. The differential nuclear and cytoplasmic localization of survivin has been shown to be due to differences in the amino-acid sequence of its carboxy-terminal domain[29]. In HCC, the predominant function of survivin is its cell cycle nuclear distribution, and not the cytoplasmic caspase-3-dependent anti-apoptotic effect[30]. So in HCC, the prognostic significance of survivin immunostain relates to the cell cycle in nuclei, and not to its cytoplasmic anti-apoptotic effect[31]. These reports suggest that most cytoplasm in the early HCC samples might be stained moderately under the conditions employed in this study.

In the present study, nuclear survivin is expressed in 64.7% of cells (median) from early-stage HCC specimens. Moreover, in all early HCCs of tumor size > 10 mm (n = 3), survivin expression was always above the median average. These data indicate that survivin could be an effective target of gene therapy for HCC, even at an early stage. Moreover, a previous study reported that in surgically removed tissues, the expression of survivin had no correlation with the patient's age, gender, tumor size and differentiation level of HCC[11]. This is consistent with the rate of nuclear expression in the small biopsy samples from early-stage HCC observed in the present study. However, a recent report showed that alpha-feto protein (AFP) blocked the X-linked inhibitor of apoptosis protein-mediated inhibition of endogenous active caspases in the cytosolic lysates of tumor cells[32]. Further immunohistological analyses of other proteins of the IAP family must be compared to the clinical parameters in early-stage HCC.

In this study, the survivin expression in HCC samples could be divided into two groups: a high-rate group (rate > 50%) and low-rate group (rate < 50%). Ikeguchi *et al*[33,34] detected survivin mRNA over-expression in 21 of 51 (41%) of HCC biopsies, and suggested that this could be useful as a prognostic factor for patients with HCC. From the early stages, the level of nuclear survivin expression may correlate with the prognosis of HCC.

HBV X and HCV core proteins activate NF-κB and/or STAT-3, which regulate the gene expression for cell survival factors such as the anti-apoptosis proteins, including survivin[35,36]. A resulting up-regulation of anti-apoptosis factors during HCV or HBV infection may contribute to hepatocarcinogenesis[35,36]. A previous report demonstrated that HBx promotes the upregulation of survivin expression in hepatoma and normal liver cells, regardless of apoptosis. These findings suggest that survivin and HBx may play important roles in the carcinogenesis of HCC[37]. Other studies have shown that HCV NS5A protein can stimulate survivin protein expression, and this may result from induced survivin gene transcription[38]. In the present study, the average nuclear survivin expression was 36.3% in hepatocyte of non-tumor specimens. This result may indicate that the hepatitis virus is associated with survivin expression in peritumoral cells. The expression of survivin has been detected in a variety of pre-neoplastic and/or benign lesions, including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis[39], suggesting that expression of survivin may occur during early malignant transformation or following a disturbance in the balance between cell proliferation and death[9]. The same process may also occur in viral hepatitis.

A previous study reported that inhibition of apoptosis by survivin plays a pivotal role in the metastasis of HCC, and it has some correlation with tumorigenesis. The expression of survivin in the primary lesion can be an indicator of metastasis and the prognosis of HCC[10]. In this study, a correlation between the survivin expression rate in the non-tumor cells and that in the HCC specimens of the high-rate group (rate > 50%) and low-rate group (rate < 50%) was observed. A significant difference in the survivin expression in the peritumoral hepatocyte was observed between the high-rate group and the low-rate group ($P < 0.01$). The survivin expression of peritumoral cells may, therefore, also be a prognostic factor for patients with HCC. Interestingly, we observed that when the amount of survivin expression was low in the adjacent non-tumor tissues, the corresponding tumor tissues also showed low expression. So in the future, when taking the target therapy of survivin into consideration, a curative effect may be possible if the amount of survivin expression with non-tumorous tissue is evaluated with liver biopsies.

Saitoh *et al*[40] demonstrated that the portal blood flow is lost before the increase in arterial flow develops in well-differentiated HCC. When the well-differentiated HCC has portal blood flow and is not hypervascular, it shows slow growth[12]. Yamaguchi *et al*[41] suggested that this phenomenon is related to hypoxia, because the well-differentiated HCC would be in a transitional stage from the portal blood supply to the arterial blood supply, but the reduction in portal flow appears prior to the increase in arterial flow. Therefore, the current research indicates that the impairment of the normal liver blood system probably causes local hypoxic regions at an early stage of hepatocarcinogensis and eventually induces angiogenesis[12]. Recent studies have shown that human solid tumors, even those less than 1 cm in diameter, may have substantial hypoxic fractions[42-43]. Hence tumor growth is restricted by limited oxygen and nutrients when they are too distant from nearby vessels[12]. Therefore, to observe the expression of survivin in early-stage HCC, HCC cells were cultured in a hypoxic environment. Previous studies have shown that survivin gene transcription is increased in hypoxic tumor cells[11]. In the current study, the hypoxic environment increased the survivin mRNA expression in both the short-term and long-term cultures. Moreover, the appearance of survivin protein is thought to control the survivin mRNA levels in the presence of anti-cancer drugs. On the contrary, in the present study, the survivin protein concentrations increased when both hypoxia and anti-cancer drugs were combined. These data suggest that survivin inhibition could therefore potentially be as effective as interventional therapy for the treatment of early HCC.

In conclusion, survivin is expressed at a rate of 64.7% (median) in early HCC. Moreover, survivin protein concentration of HCC cells increases when cultured with anti-cancer drugs under hypoxic conditions. These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality.

COMMENTS

Background

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function increases the rate of apoptosis, reduces the tumor-growth potential and sensitizes tumor cells to various chemotherapeutic drugs and γ-irradiation in both *in vitro* and *in vivo* models of various types of human tumors.

Research frontiers

Previous reports have shown survivin to be expressed in post-operative HCC tissues. However, it has not yet been fully elucidated regarding whether survivin can be used as a therapeutic target in early HCC.

Innovations and breakthrough

We studied biopsy tissue specimens to confirm the diagnosis of HCC. In all early HCCs of tumor size > 10 mm, survivin expression was always above the median average (64.7%). Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs.

Applications

These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality, such as transcatheter arterial chemoembolization (TACE), *etc.*

Terminology

In this study, a hypoxia model of cultured HCC cells was employed using an AnaeroPack for cell culture. The Anaeropack is a gas concentration-controlling reagent yielding a hypoxic atmosphere. The principal ingredient of this reagent is sodium ascorbate, which absorbs oxygen and generates carbon dioxide by oxidative degradation. The culture dishes were placed into an airtight jar with the Anaeropack and then the lid was closed. The jar was then incubated at 37℃ for 2 h. The concentration of oxygen decreased to less than 1% within 1 h and the carbon dioxide concentration was maintained at about 5% as reported previously.

Peer review

This paper investigated survivin expression in early-stage, small HCC and the results are interesting. The methods and results were clearly written, and the authors gave thoughtful discussions on this topic and their findings. This is an interesting paper that is generally well written.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153-156
2 **Bruix J**, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S179-S188
3 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750
4 **Tarnawski A**, Pai R, Chiou SK, Chai J, Chu EC. Rebamipide inhibits gastric cancer growth by targeting survivin and Aurora-B. *Biochem Biophys Res Commun* 2005; **334**: 207-212
5 **Wakana Y**, Kasuya K, Katayanagi S, Tsuchida A, Aoki T, Koyanagi Y, Ishii H, Ebihara Y. Effect of survivin on cell proliferation and apoptosis in gastric cancer. *Oncol Rep* 2002; **9**: 1213-1218
6 **Kania J**, Konturek SJ, Marlicz K, Hahn EG, Konturek PC. Expression of survivin and caspase-3 in gastric cancer. *Dig Dis Sci* 2003; **48**: 266-271
7 **Zhu XD**, Lin GJ, Qian LP, Chen ZQ. Expression of survivin in human gastric carcinoma and gastric carcinoma model of rats. *World J Gastroenterol* 2003; **9**: 1435-1438
8 **Ikeguchi M**, Liu J, Kaibara N. Expression of survivin mRNA and protein in gastric cancer cell line (MKN-45) during cisplatin treatment. *Apoptosis* 2002; **7**: 23-29
9 **Zaffaroni N**, Pennati M, Daidone MG. Survivin as a target for new anticancer interventions. *J Cell Mol Med* 2005; **9**: 360-372
10 **Zhu H**, Chen XP, Zhang WG, Luo SF, Zhang BX. Expression and significance of new inhibitor of apoptosis protein survivin

in hepatocellular carcinoma. *World J Gastroenterol* 2005; **11**: 3855-3859
11 **Yang L**, Cao Z, Li F, Post DE, Van Meir EG, Zhong H, Wood WC. Tumor-specific gene expression using the survivin promoter is further increased by hypoxia. *Gene Ther* 2004; **11**: 1215-1223
12 **Kim KR**, Moon HE, Kim KW. Hypoxia-induced angiogenesis in human hepatocellular carcinoma. *J Mol Med* 2002; **80**: 703-714
13 **Asakura T**, Ohkawa K, Takahashi N, Takada K, Inoue T, Yokoyama S. Glutathione-doxorubicin conjugate expresses potent cytotoxicity by suppression of glutathione S-transferase activity: comparison between doxorubicin-sensitive and -resistant rat hepatoma cells. *Br J Cancer* 1997; **76**: 1333-1337
14 **Thompson CB**. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**: 1456-1462
15 **Saikumar P**, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. Apoptosis: definition, mechanisms, and relevance to disease. *Am J Med* 1999; **107**: 489-506
16 **Jaattela M**. Escaping cell death: survival proteins in cancer. *Exp Cell Res* 1999; **248**: 30-43
17 **Tamm I**, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res* 1998; **58**: 5315-5320
18 **Shin S**, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, Oh BH. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry* 2001; **40**: 1117-1123
19 **Asanuma K**, Moriai R, Yajima T, Yagihashi A, Yamada M, Kobayashi D, Watanabe N. Survivin as a radioresistance factor in pancreatic cancer. *Jpn J Cancer Res* 2000; **91**: 1204-1209
20 **Asanuma K**, Kobayashi D, Furuya D, Tsuji N, Yagihashi A, Watanabe N. A role for survivin in radioresistance of pancreatic cancer cells. *Jpn J Cancer Res* 2002; **93**: 1057-1062
21 **Altieri DC**. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer* 2003; **3**: 46-54
22 **Li F**. Survivin study: what is the next wave? *J Cell Physiol* 2003; **197**: 8-29
23 **Tanaka K**, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res* 2000; **6**: 127-134
24 **Satoh K**, Kaneko K, Hirota M, Masamune A, Satoh A, Shimosegawa T. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* 2001; **92**: 271-278
25 **Kato J**, Kuwabara Y, Mitani M, Shinoda N, Sato A, Toyama T, Mitsui A, Nishiwaki T, Moriyama S, Kudo J, Fujii Y. Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. *Int J Cancer* 2001; **95**: 92-95
26 **Nakamura M**, Tsuji N, Asanuma K, Kobayashi D, Yagihashi A, Hirata K, Torigoe T, Sato N, Watanabe N. Survivin as a predictor of cis-diamminedichloroplatinum sensitivity in gastric cancer patients. *Cancer Sci* 2004; **95**: 44-51
27 **Kawasaki H**, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998; **58**: 5071-5074
28 **Sarela AI**, Macadam RC, Farmery SM, Markham AF, Guillou PJ. Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. *Gut* 2000; **46**: 645-650
29 **Grabowski P**, Kuhnel T, Muhr-Wilkenshoff F, Heine B, Stein H, Hopfner M, Germer CT, Scherubl H. Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma. *Br J Cancer* 2003; **88**: 115-119
30 **Ito T**, Shiraki K, Sugimoto K, Yamanaka T, Fujikawa K, Ito M, Takase K, Moriyama M, Kawano H, Hayashida M, Nakano T, Suzuki A. Survivin promotes cell proliferation in human hepatocellular carcinoma. *Hepatology* 2000; **31**: 1080-1085
31 **Fields AC**, Cotsonis G, Sexton D, Santoianni R, Cohen C. Survivin expression in hepatocellular carcinoma: correlation with proliferation, prognostic parameters, and outcome. *Mod Pathol* 2004; **17**: 1378-1385
32 **Dudich E**, Semenkova L, Dudich I, Denesyuk A, Tatulov E, Korpela T. Alpha-fetoprotein antagonizes X-linked inhibitor of apoptosis protein anticaspase activity and disrupts XIAP-caspase interaction. *FEBS J* 2006; **273**: 3837-3849
33 **Ikeguchi M**, Ueda T, Sakatani T, Hirooka Y, Kaibara N. Expression of survivin messenger RNA correlates with poor prognosis in patients with hepatocellular carcinoma. *Diagn Mol Pathol* 2002; **11**: 33-40
34 **Ikeguchi M**, Hirooka Y, Kaibara N. Quantitative analysis of apoptosis-related gene expression in hepatocellular carcinoma. *Cancer* 2002; **95**: 1938-1945
35 **Waris G**, Siddiqui A. Regulatory mechanisms of viral hepatitis B and C. *J Biosci* 2003; **28**: 311-321
36 **Kountouras J**, Zavos C, Chatzopoulos D. Apoptosis in hepatitis C. *J Viral Hepat* 2003; **10**: 335-342
37 **Zhang X**, Dong N, Yin L, Cai N, Ma H, You J, Zhang H, Wang H, He R, Ye L. Hepatitis B virus X protein upregulates survivin expression in hepatoma tissues. *J Med Virol* 2005; **77**: 374-381
38 **Zhou Y**, Gong GZ. Hepatitis C virus NS5A protein upregulates survivin gene expression. *Zhonghua Ganzangbing Zazhi* 2006; **14**: 414-417
39 **Altieri DC**. Survivin, versatile modulation of cell division and apoptosis in cancer. *Oncogene* 2003; **22**: 8581-8589
40 **Saitoh S**, Ikeda K, Koida I, Tsubota A, Arase Y, Chayama K, Kumada H. Serial hemodynamic measurements in well-differentiated hepatocellular carcinomas. *Hepatology* 1995; **21**: 1530-1534
41 **Yamaguchi R**, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology* 1998; **28**: 68-77
42 **Dang CV**, Semenza GL. Oncogenic alterations of metabolism. *Trends Biochem Sci* 1999; **24**: 68-72
43 **Brown JM**, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998; **58**: 1408-1416

S- Editor Liu Y **L- Editor** Kumar M **E- Editor** Yin DH

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 June 7; 13(21): 2913-2917
World Journal of Gastroenterology ISSN 1007-9327

Useful detection of CD147 (EMMPRIN) for pathological diagnosis of early hepatocellular carcinoma in needle biopsy samples

Satoshi Mamori, Keisuke Nagatsuma, Tomokazu Matsuura, Kiyoshi Ohkawa, Hiroshi Hano, Masaharu Fukunaga, Masato Matsushima, Yoshifumi Masui, Nao Fushiya, Hiroshi Onoda, Yasuyuki Searashi, Ichiro Takagi, Hisao Tagiri

Satoshi Mamori, Keisuke Nagatsuma, Yoshifumi Masui, Nao Fushiya, Hiroshi Onoda, Yasuyuki Searashi, Ichiro Takagi, Hisao Tajiri, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Tomokazu Matsuura, Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Kiyoshi Ohkawa, Department of Biochemistry 1, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Hiroshi Hano, Department of Pathology, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Masaharu Fukunaga, Department of Pathology, The Jikei University School of Medicine, The Daisan Hospital, Tokyo 105-8461, Japan
Masato Matsushima, Division of Clinical Research and development, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Correspondence to: Satoshi Mamori, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. mamori@jikei.ac.jp
Telephone: +81-3-34331111-3210 Fax: +81-3-34350569
Received: 2007-02-17 Accepted: 2007-03-21

Abstract

AIM: To make clear whether CD147 (EMMPRIN) expression in pathological tumor samples with a fine-needle aspiration biopsy is useful for pathological diagnosis of early hepatocellular carcinoma (HCC).

METHODS: Twenty-two patients (15 men and 7 women; median age 68 years, range 56-81 years) underwent a liver tissue biopsy in order to make a diagnosis of HCC. Paraffin-embedded liver biopsy tissue samples from 22 patients were stained with anti-CD147 antibody, murine monoclonal antibody 12C3 (MAb12C3) for immunohistochemical analysis. An immunohistochemical analysis of CD147 was performed and the degree of staining compared between tumor and non-tumor tissue. In addition, the degree of staining within tumor tissue was compared according to a number of clinicopathological variables.

RESULTS: The degree of staining of CD147 was significantly higher in tumor tissues than non-tumor tissues, even in tumors less than 15 mm in diameter. The expression of this protein was significantly elevated in HCC tissue specimens from patients with a low value of serum AST and γ-GTP.

CONCLUSION: CD147 serves potentially as a pathological target for cancer detection of early HCC.

Key words: CD147; Hepatocellular carcinoma; Needle biopsy

Mamori S, Nagatsuma K, Matsuura T, Ohkawa K, Hano H, Fukunaga M, Matsushima M, Masui Y, Fushiya N, Onoda H, Searashi Y, Takagi I, Tagiri H. Useful detection of CD147 (EMMPRIN) for pathological diagnosis of early hepatocellular carcinoma in needle biopsy samples. *World J Gastroenterol* 2007; 13(21): 2913-2917

http://www.wjgnet.com/1007-9327/13/2913.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, involving more than 500000 new cases yearly, with an age-adjusted incidence of 5.5-14.9 per 100000 people[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death[2]. The incidence of HCC is also increasing in Europe and the United States[3]. The early detection of tumors and development of therapies for HCC is likely to improve the prognosis[4]. Nevertheless, despite improvements in both diagnostic modalities and therapy, in many cases an accurate diagnosis still cannot be confirmed even with diagnostic imaging and the recognition of tumor markers in the serum. Particularly, hypovascular HCC which is often difficult to recognize by computed tomography (CT) requires ultrasound (US) examination for a definitive diagnosis. Tumor biopsy is an important method of evaluation in these cases, particularly in small tumors, less than 15 mm in diameter. Therefore, more sensitive tumor markers for pathological diagnosis are required.

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin, is a transmembrane glycoprotein with two immunoglobulin-like

domains. This is part of a family of proteins that includes embigin and neuroplastin[5]. Tumor cell CD147 triggers the production or release of matrix metalloproteinases in the surrounding mesenchymal cells and tumor cells, thereby contributing to tumor invasion[6-9]. A very high incidence of CD147 expression (> 80% of CD147-positive cases) is detected by immunohistochemical staining in HCC[10].

A previous paper reported a murine monoclonal antibody (MAb12C3), specific to human ovarian carcinomas was generated by immunizing mice with the human ovarian germinoma cell line (JOHY-2)[11]. In further research, using phage display libraries, MAb12C3 hybridized with the extracellular region of CD147[12]. The MAb12C3 reacted with 67.7% (21 of 31 cases) of epithelial ovarian carcinomas, but not with any of benign epithelial ovarian adenomas tested[11].

Despite extensive studies on small early stage HCCs, the morphological criteria for definite diagnosis of well-differentiated, small HCCs are still questionable[13]. This study considered whether the use of MAb12C3 against CD147 protein could recognize early stage HCC. MAb12C3 was used for an examination of antigen expression in early HCC tissue specimens and to identify any correlations between the immunohistochemical findings and the clinicopathologic characteristics of the tumors. In this study, small biopsy samples from HCC were examined with immunohistochemical staining. If significant differences are recognized between HCC with non-tumor liver tissues, CD147 may therefore be effective as a diagnostic and therapeutic target in early stage HCC.

MATERIALS AND METHODS

Patients

The study population included 22 patients (15 men and 7 women; median age 68 years, range 56-81 years) who underwent tumor and non-tumor liver tissue biopsy between January 2003 and December 2005, in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm a diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Communittee Institutional Review Broad.

Pathologic specimens

Tumor specimens were obtained by a tumor biopsy with a 21 G fine-needle aspiration kit. Non-tumorous liver tissue specimens were obtained by an 18-20 G needle liver biopsy concurrently. Formalin-fixed, paraffin-embedded specimens of liver tumors and non-tumor liver tissues were processed for conventional histologic assessment by hematoxylin and eosin (HE) staining. The tumors were histologically graded (well or moderately differentiated).

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were dewaxed and used. The specimens were stained using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system (Ventana Japan, Yokohama, Japan). A murine monoclonal antibody against CD147 protein, MAb12C3[12], was used as the primary antibody (manufactured at Department of Biochemistry 1, Jikei University School of Medicine, Japan). The antigen retrieval procedure was performed with a microwave oven in DAKO antigen retrieval solution for 30 min at 95℃ to efficiently stain the sample. The immunohistochemical staining was stronges, when performed in a microwave oven in DAKO antigen retrieval solution (Figure 1). The

Table 1 Characteristics of patients undergoing tumor biopsies ($n = 22$)

Features	Median value
Age	68 (56-81)
Sex (Male/Female)	15/7
Plt ($\times 10^3/\mu L$)	10.0 (5.1-24.5)
AST (IU/L)	68 (21-147)
ALT (IU/L)	61.5 (6-214)
T-Bil (mg/dL)	0.8 (0.4-2.3)
γ-GTP (IU/L)	48 (18-665)
AFP (ng/mL)	21.5 (3-444)
HBs Ag/HCV Ab/Others	3/18/1
Tumor size (mm)	14.5 (8-23)
Cirrhosis (positive/negative)	5/17
Differentiation (well/moderate)	15/7

Data values are expressed as the medians with ranges in parentheses unless indicated otherwise. Normal ranges: Plt (platelet count), 15-35 × 10^3/L; AST (aspartate aminotransferase), 10-33 IU/L; ALT (alanine aminotransferase), 6-35 IU/L; T-Bil (total bilirubin), 0.2-1.2 mg/dL; γ-GTP (γ-glutamyl transferase), 10-50 IU/L; AFP (α-fetoprotein), > 20 ng/mL.

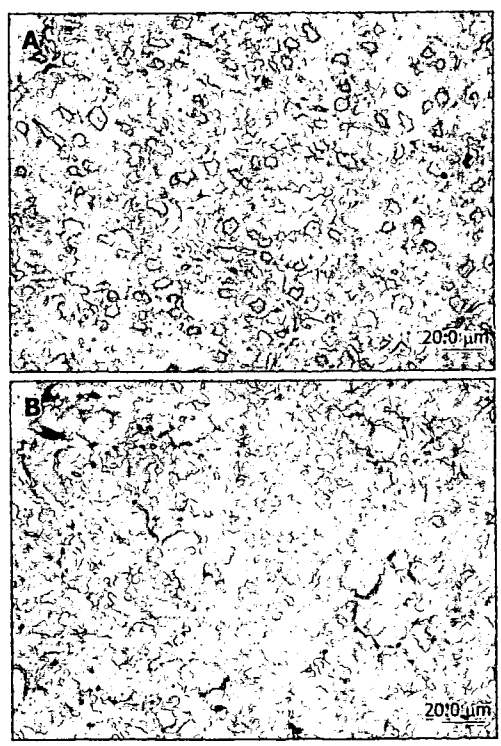

Figure 1 CD147 protein expression of HCC tissues with microwave-stimulated processing. **A**: 10 min of 10 mmol/L citrate buffer (pH 6.0); **B**: 30 min of DAKO antigen retrieval solution.

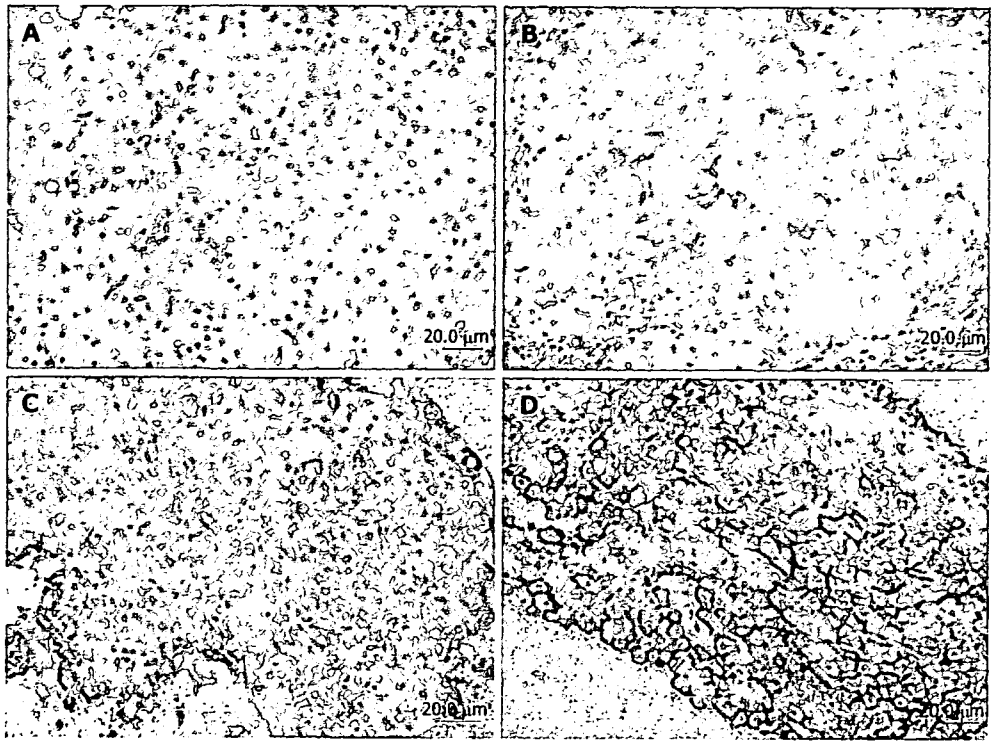

Figure 2 CD147 protein expression of non-tumor liver tissue and HCC tissue specimens. **A**: Non-tumor tissue specimen, very weak expression; **B**: Non-tumor tissue specimen, weak expression; **C**: HCC tissue, moderate expression; **D**: HCC tissue, strong expression.

Table 2 Immunohistochemical scales of tumor and non-tumor biopsy specimens

4-step scales	Tumor tissues *n* (%)	Non-tumor tissues *n* (%)	*P*
Very weak (0)	1 (4.5)	7 (31.8)	< 0.01
Weak (1)	6 (27.3)	6 (27.3)	
Moderate (2)	3 (13.6)	6 (27.3)	
Strong (3)	12 (54.5)	3 (13.6)	
Total	22 (100)	22 (100)	

sections (DAKO Cytomation, Glostrup, Denmark) were developed with 3, 3'-diaminobenzidine with 0.3% H_2O_2 and counterstained with hemotoxylin.

For each tissue sample, the fraction of the immunostained cells was recorded, and the staining intensity was estimated using a 4-step scale (0, 1, 2, 3). The tissue specimens were then initially categorized according to arbitrarily predefined criteria into 4 groups, including completely very weakly positive, strongly positive, and 2 intermediate groups. The exact criteria for these groups were as follows: very weak (1+ staining in some cells) (Scale 0); weak (1+ staining in cells) (Scale 1); moderate (2+ staining in cells) (Scale 2); strong (3+ staining in cells) (Scale 3). The examiners were blinded to patients' clinical and histological (HE staining) profile. Two investigators (H.H. and K.N.) evaluated the staining levels independently, after which any discordant evaluations were adjusted by connected microscopes and scored jointly.

Statistical analysis

Statistical analyses were performed by the Wilcoxon signed-rank test and two-sample Wilcoxon rank-sum (Mann-Whitney) test. *P*-Values < 0.05 were considered statistically significant. All these analyses were performed using STATA 9.1 (STATA Corporation, College Station, Texas, USA).

RESULTS

CD147 expression in HCC and non-tumorous liver tissue

Among all 44 tissues (22 HCC and 22 non tumorous liver tissues), CD147 immunoreactivity was detected on all cell membranes. As shown in Figure 2, CD147 was positively but weakly stained on most non-tumor liver tissues, beacause the antigenicity was activated by microwave-stimulated processing with 30 min treatment of DAKO retrieval solution (Figure 2B). However, a significant difference was observed in CD147 expression between HCC and non-tumor liver tissues (Table 2, Figure 2). In fact, there was significantly greater expression of CD147 in the carcinoma tissue specimens than in non-tumorous liver tissue specimens, including small tumors measuring less than 15 mm in size ($P < 0.05$).

CD147 expression in tumour aspirates correlates with clinical variables

Twenty-two HCC biopsy specimens were categorized into two groups for each clinical variable, above or below the median value. In these two groups, the CD147 intensity was compared. As illustrated in Figure 3, with regard to tumor size, CD147 was highly expressed in large tumors. In contrast, in the detection of serum AST and γ-GTP level, CD147 was more significant in low value groups. No significant differences were observed by other clinical parameters, such as serum AFP level. In addition, although the CD147 intensity was compared between tumor HCC