Department of Biochemistry

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Research Activities

Cancer research

1. Earlier diagnosis and therapy are the most effective methods for improvement of the prevention and the prognosis of the cancer. This year, to establish a effective, noninvasive *in vivo* system for detecting small tumors by means of molecular targeting/ imaging methods, tenascin-C, a molecule that promotes tumor-cell motility and metastasis and serves as a prognostic indicator, was selected as the target molecule for cancer detection. The ultimate goal of our study is to develop a noninvasive method of early cancer diagnosis with antibodies against tenascin-C. To achieve this goal, the usefulness of antibodies against human tenascin-C was first determined in several human cancer cell lines by means of immunocytologic studies with antibodies labeled with Alexa488. These studies showed focal distribution of intracellular tenascin-C fluorescence with fine intercellular deposits among piled-up cells rather than among cells in a monolayer sheet.

Incubation of A431 cells in a spheroid culture, a conventional 3-dimensional culture method, or in a radial flow bioreactor (RFB), another 3-dimensional culture system that provides tissue architecture and molecular function mimicking the *in vivo* environment, resulted in upregulation of tenascin-C messenger RNA and protein with simultaneous downregulation of E-cadherin and overexpression of vimentin, as in the epithelial-mesenchymal transition, with significant accumulation of transforming growth factor beta 1 in conditioned media. The sequential change of molecules related to the epithelial-mesenchymal transition was also recognized in tumor cells transplanted into nude mice. These results confirm that tenascin-C is an appropriate marker for cancer detection in an *in vivo* environment.

Clinicopathologic analysis of a large series of patients with endometrial carcinoma also showed the significant correlation of tenascin-C expression with various histopathologic risk factors for cancer. To select suitable antibodies for *in vivo* imaging, we first examined whether the fluorescence-labeling procedures reduced anti-tenascin-C antibody titers; we found that the abilities of several labeled antibodies to recognize tenascin-C decreased 10% to 30% compared with those of intact antibodies.

The tissue distribution and tumor-targeting ability of Alexa488-labeled anti-tenascin-C antibodies were analyzed in tumor-bearing nude mice. The tumor: blood ratio was significantly elevated 48 hours after administration. In *in vivo* imaging, anti-tenascin-C antibodies coupled with infrared fluorescence accumulated gradually and peaked 48 hours after administration, and the strongest fluorescent intensity was detectable. Fluorescence intensity sufficient for imaging could be detected up to 7 days later.

To produce a large quantity of antibodies, antibody-producing hybridomas were altered to proliferate under serum-free conditions, which allow the antibodies to be purified

more easily. The hybridomas produced 10 to 20 mg of functionally active antibodies within 3 weeks of culture, by means of culturing methods using either a BD Falcon CELLine CL-1000 flask (BD, Franklin Lakes, NJ) or an RFB. This project showed that antibodies against CD147 or tenascin-C were useful *in vivo* molecular targeting tools.

2. Glucose metabolism is another target for cancer chemoprevention. CD147 is the accessory subunit of a heteromeric lactate transporter, monocarboxylate transporter (MCT), which is member of the solute carrier 16 (SLC16) family of solute transporters. The MCTs transport lactate across the plasma membrane, and the CD147-MCT interaction is required for MCT activity and for trafficking to the plasma membrane. Three-bromopyruvate (3-BrPA), a pyruvate/lactate analog, is a potent glycolytic inhibitor and a candidate anticancer agent. To determine the transporters involved in the cellular influx of 3-BrPA, the role of MCTs was examined. It is of interest that resistance to 3-BrPA was found when PC-3 cells were transfected with the small interfering RNA of only MCT1 among those of the SLC16 family. PC-3 cells pretreated with MCT1 inhibitors were also resistant to 3-BrPA. Furthermore, short hairpin RNA expression vectors specific for CD147 reduced the sensitivity of PC-3 cells against 3-BrPA, suggesting that the MCT1-CD147 complex is essential for 3-BrPA uptake. Our recent study has demonstrated that 3-BrPA is transported into PC-3 cells through a CD147-MCT1 heteromeric lactate transporter complex and promotes cell death. The cytocidal activity of 3-BrPA against several cancer cell lines was enhanced under hypoxic conditions. In this microenvironment, the expression levels of CD147 and MCT1 increased compared with those under normoxic condition. These findings indicate that 3-BrPA is a potentially useful chemotherapeutic agent for hypoxic tumor cells that are preferentially resistant to chemotherapy.

3. Resistance of tumor cells to chemotherapeutic agents is a serious obstacle in cancer therapy. A conjugate of doxorubicin and glutathione *via* glutaraldehyde (GSH-DXR) strongly inhibited the glutathione *S*-transferase (GST) activity of rat hepatoma cells and all human tumor cells tested. The mechanism of action of GSH-DXR was the induction of apoptosis *via* activation of c-Jun N-terminal kinase by the binding of GSH-DXR to the active center of the GSTP1-1 enzyme, inducing cytochrome c release from mitochondria to the cytosol, caspase-3 activation, and DNA fragmentation. GSG-DXR encapsulated in an anti-CD147 IgG-coupled immunoliposome showed selective and superior anticancer effects, *via* a tumor-targeting mechanism, compared with free GSH-DXR, an empty liposome, or DXR encapsulated liposome.

4. Six cell lines with epoxomicin resistance were established. The epoxomicin-resistant cell lines are reliable tools for the therapeutic evaluation of proteasome inhibitors in preclinical trials. Moreover, these cell lines may also be useful for clarifying mechanisms of resistance to proteasome inhibitors and examining a wide variety of proteasomal functions. This year, the relation between E-cadherin expression and proteasomal inhibition was analyzed. In an epoxomicin-resistant human endometrial carcinoma cell line, Ishikawa variant, E-cadherin gene (CDH1) expression was suppressed *via* overexpression of ZEB1, a transcriptional repressor of E-cadherin. Further studies are now in progress.

5. Regulatory mechanisms of transcriptional co-activator with PDZ-binding motif (TAZ) linked to fibroblast growth factor (FGF)/receptor signaling, which plays an essential role in ossification, were determined with osteoblast-like MC3T3-E1 cells. We found that FGF-2, which inhibits bone mineralization and stimulates cell proliferation, reversibly reduced TAZ protein expression levels in MC3T3-E1 cells. A proteasome inhibitor, bortezomib, has recently been used to treat multiple myeloma. Bortezomib strongly induces the differentiation of osteoblasts and the activation of the transcription factor Runx2. However, the mechanism of osteoblast differentiation mediated by bortezomib remains unclear. Our previous report demonstrated that expression of TAZ, acting as a transcriptional coactivator of Runx2, was reduced in MC3T3-E1 cells after treatment with FGF-2. Our recent experiment has shown that bortezomib reverses the effects of FGF-2; namely, bortezomib inhibits FGF-2-induced reduction of TAZ and consequently stimulates osteogenic differentiation independently of the proteasome-inhibiting effects. These findings strongly suggest a possible mechanism for the observed bone-regenerating activity of bortezomib in patients with multiple myeloma, in addition to its anticancer activity.

Other research

1. Pharmaceutical plasma products play important roles in controlling many disorders. However, plasma products are often associated with numerous risks, such as the transmission of known and unknown infections, because they are manufactured from donated blood. The safe and efficient supply of such pharmaceutical plasma products is important. We have proposed and planned a project to produce a large quantity of plasma products with high-quality human albumin and fibrinogen using a well-defined human hepatocyte cell line cultivated in an RFB. This year, the quality and production ability of products, such as albumin and fibrinogen, were determined.
2. With methods to purify and identify ubiquitinated proteins in biological materials, several ubiquitin-protein conjugates in Tris-saline-soluble and Tris-saline-insoluble but 2% sodium dodecylsulfate (SDS)-soluble fractions were analyzed from cadmium-exposed human proximal tubular HK-2 cells. The treatment of HK-2 cells with sub-lethal concentrations of cadmium augmented water-insoluble but SDS-soluble ubiquitin-protein conjugates and rendered transcription factor STAT6 insoluble and, thus, decreased in ubiquitin target molecules in normal size in determined by SDS-PAGE. Our study confirmed that the cadmium-induced insolubilization of STAT6 was due to oxidative modifications of cysteine residues, including Cys384. These results may indicate a fundamental protective effect of STAT6 against cadmium toxicity. Therefore, structural changes in cellular proteins and the resulting loss of function are presumed to be involved in the expression of heavy metal toxicity.

Publications

Shibata S, Marushima H, Asakura T, Matsuura T, Eda H, Aoki K, Matsudaira H, Ueda K, Ohkawa K. Three-dimensional culture using a radial flow bioreactor induces matrix metalloprotease 7-mediated EMT-like process in tumor cells via TGFbeta1/Smad pathway. *Int J Oncol* 2009; **34:** 1433–48.

Matsudaira H, Asakura T, Aoki K, Searashi Y,

Matsuura T, Nakajima H, Tajiri H, Ohkawa K. Target chemotherapy of anti-CD147 antibody-labeled liposome encapsulated GSH-DXR conjugate on CD147 highly expressed carcinoma cells. *Int J Oncol* 2010; **36:** 77–83.

Eda H, Aoki K, Kato S, Okawa Y, Takada K, Tanaka T, Marumo K, Ohkawa K. The proteasome inhibitor bortezomib inhibits FGF-2-induced reduction of TAZ levels in osteoblast-like cells. *Eur J Haematol* 2010; **85:** 68–75.

Janer A, Werner A, Takahashi-Fujigasaki J, Daret A, Fujigasaki H, Takada K, Duyckaerts C, Brice A, Dejean A, Sittler A. SUMOylation attenuates the aggregation propensity and cellular toxicity of the polyglutamine expanded ataxin-7. *Hum Mol Genet* 2009; **19:** 181–95.