S-5

Carcinogenesis and Toxicology Bioassays Using Transgenic Mice in Japan -a review of MHW Kurokawa Group Study and recent NIHS efforts -

Jun Kanno and Yuji Kurokawa

Division of Cellular and Molecular Toxicology,
Biological Safety Research Center,
National Institute of Health Sciences

In our Research Group (Chair: Dr.Kurokawa) funded by MHW, Japan and in our Division of Cellular and Molecular Toxicology, we are developing and evaluating methods for faster and more accurate carcinogenesis bioassay systems as well as model systems for molecular biology-based toxicological studies by using biotech recombinant mice. Today, I would like to present a review of these efforts with a brief discussion made during the process.

When we consider using these Biotech-Recombinant Mice/Rats in Short-term Assay for Carcinogenicity, there are several questions to be answered.

As far as the GENES are concerned:

- What kind of genes should be focused?
- Recombinant-mice now available are sufficient?
- Is there a single transgenic (TG) or knockout (KO) strain that covers all?
- Are they good for detecting cancer promoters?

About ORGAN SPECIFICITY:

- Are target organs similar to that of the parental wild mouse?
- Will tumor type depend on the transgenic or Knocked out gene?

When RAPIDITY & RESOLUTION are compared with conventional bioassay system, we already know these mice tend to develop tumors faster, but there still are questions like,

- How fast the tumors appear and how rapid they grow?
- How sensitive to carcinogens?
- Can we get clearer resolution between treated & control?
- How should we set the exposure period not to get saturation and to get best resolution?

To answer the questions regarding the GENES, in our group, several ras-modified strains as shown here have been studied. With Dr. Yamamoto at Central Institute of Experimental Animals, we have finished carcinogenicity studies on 18 genotoxic chemicals and 6 non-genotoxic chemicals using c-H-ras BALB/c-C57B26F1 mice, so-called rasH2 mice.

What we've found in a series of *ras* transgenic animals was the redundancy of the *ras* genes. Dr. Katsuki looked into the function of K-, N-, and H-*ras* in mouse, not only by making KO mouse of each *ras* gene but also generating compound mice of them, that is double and triple KO mice. Among these three types of single *ras* KO mice, only K-*ras* (-/-) was lethal, and K-ras(+/-) was viable. Cardiac rupture due to hypogenesis of cardiac wall was the cause of lethality in the K-ras (-/-) mice.

When compound mice from these three single *ras* KO mice were generated, functional redundancy was found, that is, K-ras (-/-) mouse was fatal, and K-ras (-/+) mouse was viable when at least one copy of N-ras co-existed. In short, one copy of K-ras and one copy of N-ras are sufficient for viability. These data show that ras family has mutual redundancy. Addition to these findings, Dr. Katsuki's group has examined the carcinogenic properties of ras-KO mice. They found that H-ras KO mouse has lower tumorigenicity.

Then, how about the function of the human c-H-ras introduced in mice. Dr. Wakana has tested several carcinogenic chemicals on such mouse system. He found that, although some exceptions, there was a tendency that human c-H-ras TG mice induced higher frequency of tumor with higher multiplicity than non-TG wild mice did. When the ras-gene mutation was analyzed, the mutation of human c-H-ras was present in roughly 10 to 20% of the tumors developed in the TG mice. However, the mutation of endogenous (mouse) K-ras was not found in the tumor in the TG mice. On the other hand, the mutation of endogenous K-ras was found in almost all the tumors of non-TG mice.

These findings leads to the assumptions that 1) ras mutation is not directly related to carcinogenesis in this system, and 2) the treatment may have induced ras-overload in this system, because K-ras mutation in tumors of non-TG mice was detected in a high frequency.

Then the question would be what is the mechanism of high tumor yield in this human c-Ha-ras TG mouse. The TG mouse tumors had very high frequency of loss of heterozygosity (LOH) whereas non-TG mice had virtually no LOH. Although further analysis is needed, this result suggests that LOH might have induced genomic instability. A rat system carrying human c-H-ras (Dr. Tsuda) showed high tumorigenicity. This rat was exposed to MNU and the development of mammary tumors was monitored. The tumor yield was higher in TG rats in all three parameters, such as incidence, multiplicity and average size. Although the LOH was not analyzed because of the rat system, we assume that the events similar to mouse had also taken place in this system.

Next, I would like to briefly talk about p53 KO mouse. It is known that p53 KO mouse shows, at least, impairment of DNA repair and accelerated cell cycling. And these two characters are assumed to contribute to the high carcinogenic responses. According to Dr. Ishikawa, transplacental exposure of ethylnitrosourea

leaded to a rapid development of brain tumor in p53(-/-) mice.

These are the examples of rapid and sensitive response of TG/KO mice to complete (or genotoxic) carcinogens. However, in contrast, some cancer promoters (or non-genotoxic carcinogens) failed to induce clear response in TG or KO mouse systems. We are conducting several trials including application of 2-stage model to the TG and KO mouse systems.

The last issue here in carcinogenesis bioassay is about the rapidity and resolution. We already know that most TG/KO systems develop tumors fast, and if two-stage model is applied, some system develops tumors in 6 weeks. However, we need to study more to get better resolution, especially we need to know how long we should expose the animals to the chemicals.

As a second topic, I would like to briefly introduce two examples of recent studies using TG/KO mouse systems in our division, hoping that these studies may indicate new viewpoint on molecular biology-based toxicity studies. One is about hemopoietic systems, and another is about endocrine disrupting chemicals.

In our lab, Dr. Hirabayashi has established several transgenic strains related to IL-3 receptor systems. One of such strain is a mouse that is overexpressing human IL-3 receptor (WD2 mouse). IL-3 is a cytokine so-called multistimulating factor for hemopoiesis. This IL-3 differs among spices and human IL-3 has no effect on the mouse receptor. As expected, this WD2 mouse responds to both human IL-3 and murine IL-3, whereas wild type mouse only responds to murine IL-3. The response to IL-3 is monitored by the incidence of colony formation *in vitro* using bone marrow cells (One colony is considered to have derived from one hemopoietic stem/progenitor cell in the marrow). Various types of colonies are induced, such as granulocyte, monocytic, mast cell, mixed (granulocyte/monocyte, granulocyte/monocyte/megakaryocytic) and a compact one mimicing a stem cell colony. This compact colony appeared at about one fourth of total colony number. She further characterized the compact colony by re-plating with either human IL-3 or murine IL-3 in the culture system. A variety of colonies had responded to murine IL-3. However, interestingly, the compact colony had reacted only to human IL-3 and proliferated. The compact coloy was found to be positive for B220, negative for Gr-1 and Thy-1, indicating a B-cell lineage. Further analysis showed that, human IL-3 induced self-renewal of the colony, whereas murine IL-7 induced differentiation toward mature B-cell, and once differentiated, no colony could be reproduced by IL-3.

Eventhough this system turned out to be the B-cell progenitor, not the hemopoietic stem cell it self, this can be a good model to see, 1) self-renewal and differentiation ability, and 2) its manipulation by species specific cytokines. Using these unique characters, this system may serve as a model that can distinguish the effect of chemicals on clonal renewal from differentiation. This distinction has merit because the damage to stem cell renewal may be long lasting or even irreversible whereas the damage to differentiation may be short-lived and reversible, and yet these two types of damage can be phenotypically identical in traditional short-term toxicity assay.

Next example is the Endocrine Disruptor. Application of TG/KO mouse system to this subject has been

considered in our laboratory since the issue came out. One trial is to utilize the p53 KO mouse system, expecting the growth advantage of target organs by accelerated cell cycling and/or lower apoptotic tendency. In a preliminary study, the uterus of the ovariectomized p53 (-/+) mouse showed a tendency of growth advantage under strong estrogen stimulation. However, this study definitely needs further validation especially in low-dose exposure. At least, there is a possibility that some TG/KO mouse systems may have different sensitivity to different receptor-mediated growth and/or differentiation signals.

When the target is expanded to general toxicology, The TG/KO animal system forces us to think over about the toxicity on signal transduction systems. For example, both intrinsic and extrinsic stimuli may produce a very similar effect, simply because the secondary messengers are common; distinction between normal reaction and adverse effect is not clear at a certain level of signaling events. According to this, while the receptor-ligand interaction can be theoretically monitored in the *in vitro* system with a virtually non-threshold dose-response, we don't know yet how low dose effect can be detected as an adverse effect in the *in vivo* system. Therefore, unless the molecular mechanisms are thoroughly known, *in vivo* experiments are as essential as *in vitro* experiments for identification of the endpoint of toxicity. Such mutual supplementation of *in vitro* and *in vivo* systems is important for the total understanding of toxicology, at least for a while.