U.S.-Japan Seminar on "Genomic Instability during Carcinogenesis and Tumor Progression"

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The recent remarkable progress in molecular genetical studies on cancer has made it clear that carcinogenesis and tumor progression are caused by accumulation of multiple chromosomal alterations and mutations in protooncogenes and tumor-suppressor genes. The aim of this conference was to discuss the most recent knowledge on the molecular mechanisms leading to genetic alterations during the course of carcinogenesis and tumor progression. It is strongly suggested that genomic instability is one underlying mechanism for the accumulation. Participants in the conference, that was held on February 15-17, 1992 at Kauai, Hawaii and sponsored by the U.S.-Japan Cooperative Cancer Research Program, were leading scientists in the fields of cell cycle control, mitosis, recombination, mutation and gene amplification. The organizers of this conference were Curtis C. Harris (National Cancer Institute, Bethesda) and Setsuo Hirohashi (National Cancer Center Research Institute, Tokyo).

CELL CYCLE CONTROL AND ANEUPLOIDY

The current knowledge on the biology of the mitotic apparatus was reviewed by Bill R. Brinkley (Baylor College of Medicine, Houston). A molecular scheme of the centromere-kinetochore complex in animal cells was presented. Using sera from patients with autoimmune disorder CREST syndrome, 50-60% of whom have anti-centromere antibodies, a family of centromeric proteins has been identified. Among them, CENP-B, an 80-kD acidic protein with DNA-binding ability, was demonstrated to bind with a 17-base-pair DNA motif which is located within the centromeric repetitive DNA sequence of each chromatid. CENP-B also binds with certain microtubule-associated proteins (MAPs), and as a result, CENP-B and MAPs formed a bridge between centromere of a metaphase chromosome and mitotic spindle microtubules. Progress in characterizing the function of centromeric proteins was also presented. He also discussed the mechanism of asbestos-induced aneuploidy in human cells. Asbestos fibers, endocytosed by cultured human lung cells, caused a precipitous increase in

mitotic spindle aberrations, micronuclei and multinucleated cells within two to five days after exposure. More significantly, when crocidolite and chrysotile fibers were incubated with whole-cell homogenates from the same cells, selective binding of several important spindle proteins, including tubulin, actin and centromeric proteins, was demonstrated by SDS-PAGE and Western blotting. The selective affinity of asbestos fibers for mitotic spindle proteins could account for their targeting to the mitotic apparatus and induction of aneuploidy leading to the development of malignant human mesotheliomas.

Hiroto Okayama (Research Institute for Microbial Diseases, Osaka University, Osaka) discussed the dysregulation of human mitotic control genes in cancer cells and its implication for their genetic instability. The G2 phase of the cell cycle is the period in which entry into mitosis is regulated by a complex system involving *cdc2*⁺ protein kinase as a key controller. In fission yeast, the activity of *cdc2*⁺ kinase is regulated by the *cdc25*⁺ phosphotyrosine phosphatase and *wee1*⁺/*mik1*⁺ tyrosine kinases, and aberrant expression of these mitotic control genes, such as overexpression of *cdc25*⁺ combined with underexpression of *wee1*⁺, disturbs coordinated cell division and prematurely induces mitosis, resulting in the generation of anucleate and aneuploid cells.

This regulatory system is evolutionarily well-conserved throughout higher eukaryotes. *String* is a *Drosophila* homolog of *cdc25*⁺ and CDC25Hs (CDC25HuA) is a human homolog isolated by Sadhu, K., *et al.* (Proc. Natl. Acad. Sci. USA, 87; 5139-5143, 1990). Recently a *wee1*⁺ homolog (WEE1Hu) and two additional *cdc25*⁺ homologs [CDC25HuB and CDC25HuC] from human cells were cloned in Dr. Okayama's laboratory. CDC25HuA and CDC25HuB were predominantly expressed in mammalian cells during G2, sharing remarkable similarity with the *cdc25*⁺ gene in yeast. In most of the mammalian cells examined, CDC25HuB was expressed 10- to 100-fold more than CDC25HuA. The amount of CDC25HuB mRNA in the steady state was particularly high in various cancer cells and SV40-transformed cells, with a noticeable correlation between the extent of aneuploidy and the level of its expression. Moreover, the expression of CDC25HuA, CDC25HuB and *cdc2* was no longer

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cell cycle-dependent in the SV40-transformed cells, and the aberrant expression of these mitotic control genes occurred immediately after transformation of human fibroblasts by SV40.

Takeharu Nishimoto (Graduate School of Medical Science, Kyushu University, Fukuoka) discussed the RCC1 gene which was isolated by complementing the tsBN2 mutation, a temperature-sensitive mutation of the BHK21 hamster cell line. The RCC1 gene encodes a 45 kD nuclear DNA-binding protein and appears to be essential in sequential coupling of DNA replication and mitosis. Upon loss of RCC1 protein, tsBN2 cells which are replicating enter the mitotic phase. Recently, Bischoff, F. R. and Ponstingl, H. (Nature, 354; 80-82, 1991) found that RCC1 protein binds to the small G protein, i.e., Ran, and has a catalytic activity of guanine nucleotide exchange in this protein. Thus, the RCC1/Ran system seems to be involved in the GTP cycle in the nuclei. Nishimoto and his co-workers proposed that the DNA-binding activity of RCC1 plays a role in the signal transduction from DNA to the regulator of MPF (maturation promoting factor). However, the RCC1 protein without the DNA-binding domain complemented tsBN2 mutation efficiently and the endogenous RCC1 disappeared in these ts⁺ transformants of tsBN2 cells transfected with the deleted RCC1 cDNA at 39.5°C. Although the majority of the deleted RCC1 protein was in the cytoplasm, a significant amount of the deleted protein was kept in the nuclei and co-migrated with the nucleosome fraction in sucrose-density gradient analysis. Since the deleted RCC1 protein did not bind to DNA by itself, these results indicated that RCC1 protein binds to the chromatin with the aid of other unknown protein(s).

Since premature chromosome condensation should cause chromosomal loss, the defect of the RCC1/Ran system may promote carcinogenesis. It would be interesting to investigate the RCC1/Ran system in cancer cells.

Human cancers such as colorectal carcinomas are known to exhibit reduction of telomeric sequences. A chromosome with such terminal deletion tends to generate a reactive end that may lead to additional chromosomal aberrations upon cell division. These aberrations are a characteristic of cancer cells, and telomere reduction is thought to contribute to carcinogenesis and subsequent tumor progression.

Ryo Kominami (Niigata University, School of Medicine, Niigata) reported that, in addition to the reduction of telomeres, size-variants are frequently observed in tumors and in murine cell lines. This indicates that telomeres are highly unstable in both tumors and certain cultured cell lines. Since the changes were detected as discrete bands, they were probably caused by recombination between telomeres. He also described conditions in which telomere elongation could occur by the *de novo*

synthesis of a telomere by the ribonucleoprotein enzyme telomerase.

Gregg B. Morin (University of California, Davis) summarized DNA recognition properties of human telomerase and its role in carcinogenesis. Human telomerase, *in vitro*, recognizes the truncated chromosome end and adds telomeric repeats. The DNA sequence requirements for telomerase recognition of the DNA primer are minimal. Since telomerase may not be continuously expressed in somatic cells, it must be activated to contribute to carcinogenesis. The importance of telomere changes in chromosome instability has been appreciated but its exact mechanistic role still remains unclear.

GENE MUTATION AND AMPLIFICATIONS

Patterns of spontaneous mutations in experimental systems and patterns of mutations in cancers were compared by Lawrence A. Loeb (Gottstein Memorial Laboratory, University of Washington, Seattle). Evidence indicates that the point mutation frequency in somatic cells is 1.4×10^{-10} mutations/nucleotide/cell generation. Three potential sources of spontaneous mutations, 1) chemical hydrolysis of the glycosylic bond in DNA; 2) damage to DNA by oxygen free radicals; and 3) misincorporations by DNA polymerases, were analyzed. Each process, if not repaired, could generate about 3000 mutations/cell/day. Spontaneous chemical hydrolysis of DNA generates abasic sites that direct the preferential incorporation of deoxyadenosine. Replication of DNA damaged by oxygen free radicals results in a spectrum of nonrandom single-base mutations, most frequently C-T and G-T substitutions. In addition, an unusual mutation, two adjacent thymidines substituting for two cytidines, is produced by either oxygen free radicals or UV irradiation. Infidelity of different DNA polymerases yields a characteristic pattern of single base substitutions, predominantly transitions. A comparison of these data with the spectrum of spontaneous mutations that have been analyzed in different laboratories indicates that abasic sites are not a major premutagenic event and favor replication errors or oxygen free radical damage as major sources of spontaneous mutations.

Hiroyasu Esumi (National Cancer Center Research Institute, Tokyo) reported an unusual finding on altered specificity of mRNA splicing in analbuminemic rat liver during carcinogenesis and aging. In analbuminemic rats, a seven-base-pair deletion in the splice-donor site of the 9th intron of albumin gene blocks normal albumin mRNA splicing, resulting in production of 9th-exon-skipped albumin mRNA. Only a trace amount of albumin synthesis is detected in the liver of analbuminemic rats and few hepatocytes are positive for albumin immunohistochemically at 4 weeks after birth. However, a

large number of hepatocytes became albumin-positive after administration of hepatocarcinogens such as 3'-MeDAB, Glu-P-1 and AAF or during aging. Western blot analysis of the liver of aged or carcinogen-treated albuminemic rats showed that small-molecular-weight anti-albumin reactive proteins which are not present in the younger untreated rat liver are present. Subcellular localization of the albumin-related proteins was found to be the particulate fraction, in which albumin is not found normally. Northern blot analysis revealed that small-sized mRNA increased during the course of carcinogenesis and aging and those mRNAs were found to be 9th and 10th exons-skipped mRNAs. The fate of albumin-related proteins translated from aberrantly spliced mRNA was examined by expressing these mRNAs in various hepatoma cells. The results showed that truncated albumin due to the 9th and 10th exon deletion accumulated in the particulate fraction of the cells. He concluded that alteration of the specificity in mRNA splice site selection resulted in an ectopic accumulation of aberrantly synthesized protein. Not only the structural stability of the gene but also functional stability of RNA processing is important in changes of phenotypic expression during carcinogenesis.

Gene amplification is frequently observed in tumors and transformed cell lines, and is one marker for genomic instability. Thea D. Tlsty (University of North Carolina, Chapel Hill) reported a correlation between the ability of cells to amplify endogenous genes and their ability to form a tumor when injected in the appropriate animal. Human cells were exposed to N-(phosphonoacetyl)-L-aspartate (PALA), which specifically inhibits aspartate transcarbamylase activity of the CAD enzyme, and PALA-resistant subclones were selected. She observed a striking parallel between the ability of these cell lines to become resistant and the ability of the same cell lines to form tumors after injection into day-old syngeneic rats. Molecular analyses of PALA-resistant subclones revealed that this resistance was due to amplification of the CAD gene. Tumorigenic cell lines demonstrate a frequency of gene amplification that can be greater than 100 times that seen in non-tumorigenic cell lines. In contrast, gene amplification is undetectable in primary, diploid human cell populations. These results demonstrated that a dramatic difference exists between primary diploid cell populations and immortalized populations in their ability to amplify genomic sequences and suggested a significant difference in genetic instability between these two cell types. An approach using somatic cell hybrids to identify the gene(s) controlling the susceptibility to gene amplification was also discussed.

The genes that encode the variable regions of immunoglobulins and T-cell receptors are assembled by a common recombination system referred to as VDJ re-

combination, which is probably controlled by the RAG-1 and RAG-2 genes. Frederick W. Alt (Howard Hughes Medical Institute, Columbia University, New York) has investigated the function of the RAG-2 gene by constructing RAG-2-deficient transgenic mice. Such mice are viable but show a severe combined immune deficiency (SCID) due to a total inability to initiate VDJ recombination. RAG-2-deficient mice have no obvious defect in any tissue or lineage other than lymphocytes, and thus he concluded that VDJ recombinase activity and RAG-2 gene function is required only for lymphocyte development. Since homozygous SCID mutants also have a defect in general DNA repair processes, he is currently isolating genes which complement the defects of both DNA repair and VDJ recombination. Such genes are likely to be candidate genes for genomic instability in general.

TUMOR SUPPRESSOR GENES

Curtis C. Harris (National Cancer Institute, Bethesda) reviewed involvement of p53 tumor suppressor gene in human carcinogenesis. Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues and hemopoietic tissues. Analysis of these mutations can provide clues to the etiology of these diverse tumors and to the function of specific regions of p53. Transitions predominate in colon, brain and lymphoid malignancies, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the lung, breast and liver. Mutations at A:T base pairs are seen more frequently in esophageal than in other solid tumors. Most mutations in lung, breast, and esophageal carcinomas are dispersed among numerous evolutionarily conserved codons. In liver tumors in persons from geographic areas in which aflatoxin B1 and hepatitis B virus are cancer risk factors, most mutations are at one nucleotide pair of codon 249. One hypothesis is that aflatoxin B1 is metabolically activated to form the promutagenic N7dG adduct and enhanced cell proliferation due to chronic active viral hepatitis allows both fixation of the G-to-T transversion in codon 249 of the p53 gene and selective clonal expansion of the cells containing this mutant p53 gene. Induction of skin carcinoma by ultraviolet light is indicated by the occurrence of p53 mutations at dipyrimidine sites, including CC to TT double base changes. Therefore, these differences in p53 mutational spectra among human cancer types reflect the etiological contributions of both exogenous and endogenous factors to human carcinogenesis and, secondly, the selective clonal expansion ad-

vantages of various mutant p53 genes in different human cell types.

Jun Yokota (National Cancer Center Research Institute, Tokyo) described the presence of multiple genetic alterations in human lung and colorectal carcinomas. The purpose of this study was to clarify the molecular mechanisms of tumor progression. In lung carcinoma, inactivation of the RB and p53 genes as well as allelic losses on chromosome 3p were detected frequently in primary tumors. In addition to these genetic alterations, accumulation of chromosome 11p allelic deletions and *myc* amplification in the process of metastasis were observed in some cases. In colorectal carcinoma, alterations of the p53 and DCC genes were detected in approximately 70% of primary tumors and in 100% of liver metastases. Furthermore, allelic losses on chromosomes 13q, 14q and 18q accumulated during tumor progression. Among several genetic alterations, p53 alterations seem to occur relatively early in tumor progression and may be critical events for the malignant conversion of pre-malignant cells. The results suggest that at least three to five genes are mutated in tumor cells with metastatic ability, and that the p53 gene would be a trigger for genomic instability occurring *in vivo* during tumor progression.

SV40 T antigen binds and thereby inactivates p53, and it is well known that cultured SV40-transformed cells develop marked chromosomal aberrations. Michael A. Tainsky (University of Texas M.D. Anderson Cancer Center, Houston) reported that fibroblasts from patients with Li-Fraumeni syndrome, who possess germline mutations of p53, acquired an altered morphology, chromosomal anomalies and the ability to exhibit anchorage-independent growth during culture *in vitro*. Unlike normal human fibroblasts, these cells yielded established cell lines. These findings suggest that p53 might be involved in the regulation of the expression of various mitotic control genes, and that inactivation of p53 by mutations might result in their aberrant expressions, thereby inducing genetic instability.

Setsuo Hirohashi (National Cancer Center Research Institute, Tokyo) presented data on alterations of tumor DNA ploidy and tumor suppressor genes in human cancer progression. Inactivation of multiple tumor suppressor genes plays an important role in tumor progression, and directly or indirectly causes chromosomal and genetic instability that leads to the emergence of more malignant clones. Hepatocellular carcinomas (HCCs), in which morphological evidence of stepwise progression is clearly observed, were selected for study. In liver damaged by virus infection, small nodular lesions develop as a clonal expansion of hepatocytes. Within these nodules, HCCs of the moderately or poorly differentiated type often emerge as a subclonal expansion and form nodule-in-nodule lesions. The ordinary HCCs formed by progression show greatly increased cell proliferation, increased number of nuclear organizing regions (NORs) and aneuploidy in some tumors, together with inactivation of multiple tumor suppressor genes including p53 and RB. Association between aneuploidy and p53 mutation was observed not only in HCCs but also in other cancers such as gastric cancer and ovarian cancer. Furthermore, Yoshinori Murakami (National Cancer Center Research Institute, Tokyo), who attended as an observer, added data of a close association of the presence of p53 mutation with chromosomal aberrations in cultured human carcinoma cell lines.

Thus, the most recent information on: a) genetic and chromosomal alterations in cancer; b) their role in carcinogenesis and tumor progression; c) the role of genomic instability in accumulating such alterations; and d) fundamental mechanisms possibly involved in genomic instability were exchanged. Although this is a relatively new field in cancer research, the participants predicted that there will be rapid progress in this field in disclosing the mechanisms of genomic instability occurring in cancers and their relation to the multiple genetic and chromosomal alterations observed during carcinogenesis.

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