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26
Toxicogenomics: Japanese Initiative

Tetsuro Urushidani and Taku Nagao

26.1
The Present State of Drug Development Genome Science

The human genome project, which started in 1990, was a great milestone in the history of science that revealed the entire genetic blueprint of the human being [1]. Simultaneously, it is said that it activated the economy of the U.S.A. and saved it from economic crisis. People outside the U.S.A. missed their chance to join the game earlier, when it was unrecognized that the gene itself would become a business opportunity. The Japanese government started its 'millennium plan' in 2000 to support and promote gene research, especially as related to five serious diseases – dementia, cancer, hypertension, diabetes, and allergy. However, it was somewhat too late.

In addition to support by the government, participation of private capital is indispensable for the development of scientific research. Although attention has only recently been directed to business based on genome science, presently, the movement of investors is slow and investment is not well focused. There was optimism at the beginning of the human genome project that development of remedies would immediately be possible when genomic information related to a certain disease was revealed. Today, however, everybody realizes that that was an illusion. However, every time a disease-related gene is identified or assigned, the news is always released with a comment that a medication for the disease will soon appear. This is a great misunderstanding. Even in the days when the human genome was barely sequenced, a large number of diseases caused by genes had already been identified. For example, in 1989 the causal gene of cystic fibrosis was found to be CFTR [2], and this fact in itself brought about no change in the therapy of this disease, and of course, cystic fibrosis is still incurable today. Similarly, it could be said that the analysis of familial Alzheimer's disease did not contribute to the clinical development of donepedil at all. However, it is not true that elucidation of the human genome contributes little to drug development. On the contrary, it is a powerful and efficient tool for producing a candidate compound in combination with high-throughput chemical synthesis and screening systems, once a target molecule is decided upon. This is why drug manufacturers around the world compete for the use of genome information. Will this strategy really accelerate drug discovery?

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In the worldwide pharmaceutical market, as well as in Japan, the entry of new chemicals tends to be decreasing, in spite of the progress of technology in finding candidates for new drugs. Some reasons for this are that medical requirements have matured in certain fields and clinical trials are more difficult to perform, but the main reason appears to be discrepancies between preclinical and clinical results, which needs to be worked on. The possibility for a newly found chemical to be successfully developed as a medicine is quite low, even if it is perfectly aimed at its molecular target. According to the ex-president of GlaxoSmithKline, the success rate is lower than 1 in 10 000 [3]. Figure 26.1 displays the success rate of new chemical compounds as investigated by a committee of the Pharmaceutical Manufacturers Association in Japan [4], showing a very low success rate (63 out of 238) even for chemicals that were selected from among more than 400 000 candidates. If the number of chemical compounds that succeed as a medicine is proportional to the number of developed chemicals times a reasonable factor, a small number of large manufacturers with a huge scale of development should be able to monopolize the worldwide medical market. Even if the chance of success is too low to keep many projects going at once, there nevertheless is a chance for medium-scale companies, like those in Japan, to create a 'big' product. It is necessary for the pharmaceutical companies in Japan to figure out how they can successfully develop candidate chemicals into medicines so that they can survive in the 21st-century world market.

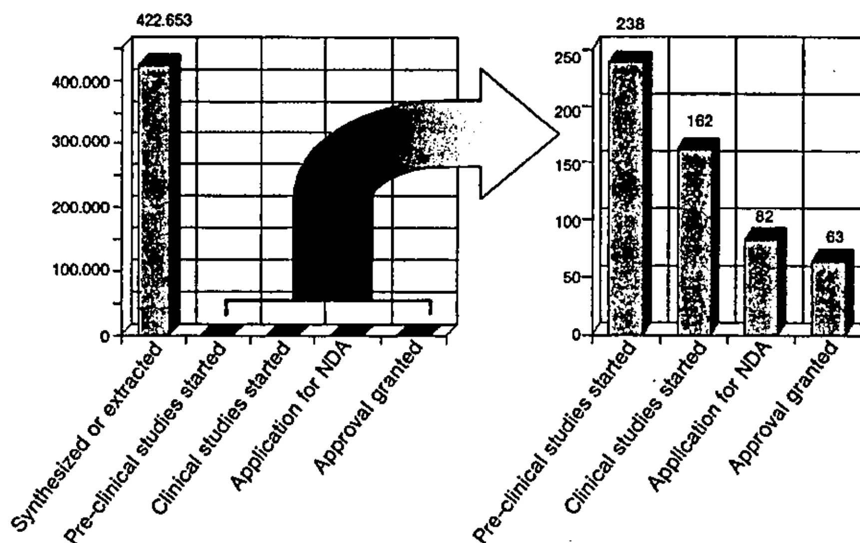

Fig. 26.1 Success rate of drug development in Japan (1996–2000). Data are taken from the report by the research and development working group of the Japan Pharmaceutical Manufacturers Association [4]. *Left panel:* Among 422 653 synthesized or extracted chemicals, fewer than 250 were examined in preclinical studies. *Right panel:* Expanded view of the last four bars in the left panel. Note that the success rate was 26.5% (63 out of 238), even for chemicals selected from the 422 653 starting chemicals and that the overall success rate was only 1 in 6709 or 0.015%.

26.2
The Necessity of Toxicogenomics

Many candidate drugs drop out in the stage of preclinical and clinical testing. A considerable proportion of the dropouts are related to toxicity (Figure 26.2), even in the statistics of 1997 [5]. Today, it is common sense that the appropriate molecular target to select is the human type. For example, in development of a drug acting on a 7-transmembrane receptor, cloned human-type receptor is used for screening. Previously, when preclinical screening was performed in laboratory animals, ineffectiveness due to species differences in the structure of the target molecule often emerged during clinical trials, but today, such an event does not happen. However, 'nonpredictable' deleterious effects or toxicity cannot be overcome by this strategy. In contrast to pharmacological effects, drug toxicity effectively happens in a black box and cannot be satisfactorily predicted in preclinical experiments. In some extreme cases, serious adverse effects emerge even after the drugs are widely distributed on the world market. A top priority should be the solution of this paradox, i.e., how to predict 'nonpredictable' toxicity. To do this, toxicogenomics is considered to be one of the most powerful strategies.

In classical toxicology, toxicity was mainly designated and assessed according to pathological changes observed in a certain organ as a result of a chemical administered in an excessive amount. In the clinical field, serious adverse effects in humans cannot be allowed, even they occur rarely. For this kind of situation, biostatistics, based on the incidence of a certain phenotype observed in limited numbers of animals that received a clinically meaningful dose, is useless because, to see reasonable numbers from toxic effects it is necessary to increase the animal dose to much higher than the clinical dose range. In preclinical tests, therefore, toxicologists have been obliged to extrapolate from data based on pathological changes observed in laboratory animals that received high, sometimes unreasonable, doses to the toxicity found with low incidence at the clinical dosage. There is, however, no assurance that these two phenomena are biologically related. The response of an or-

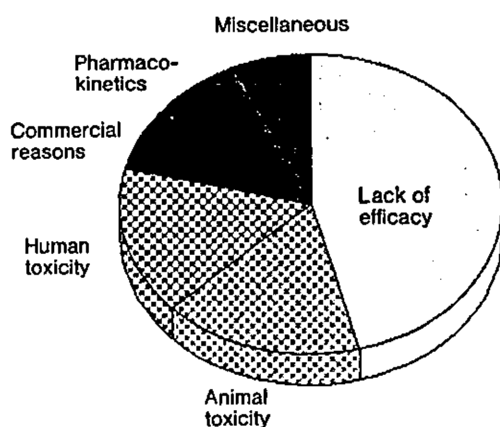

Fig. 26.2 Reasons for failure of 121 new chemicals in clinical development. Data published by the Center for Medicines Research were taken from the review by Kennedy [5]. Of 198 compounds, 77 anti-infective drugs were excluded since most of them were terminated because of unsatisfactory pharmacokinetics.

ganism to a toxicant at low dose that subsequently causes pathological changes in certain organs should be detectable as changes in gene expression, protein synthesis, metabolism, etc. Of these, the expression of genes, or the amount of mRNA, is the most sensitive measure and is one of the greatest advantages of the technology of genomics.

Although genomics, which comprehensively analyzes all the expressed genes, and proteomics, which comprehensively analyzes all the existing proteins, are powerful techniques, they have limitations. Especially when they are used for elucidating the causal factor(s) in a certain disease or for estimating the pharmacological effects of a certain drug, one often encounters a difficulty in extracting meaning from the enormous amount of information. In contrast, this 'omic' analysis is rather suitable to forecasting the 'unpredictable' response of a black box. For example, an 'omic' analysis of a certain disease starts with a comprehensive analysis, but the aim is to find biomarker(s) specific for the disease; in other words, the ideal goal is that the symptoms of the disease become explainable by one or a few changes in genes or proteins. On the other hand, it is theoretically unlikely that predicting the toxicity of various chemical substances can be based on a few biomarkers. Of course, one may expect that a small number of gene clusters that represent the property of a group with a common toxicological mechanism can be identified, and in fact, some successes have been reported [6]. However, needless to say, one should start with a comprehensive analysis, especially when predicting the toxicity of a new chemical entity.

26.3 Toxicogenomics Project 2002–2007

26.3.1 Planning Process and the Present Organization

As described in the previous section, the Japanese pharmaceutical industry and the Japanese government both recognized that predicting toxicity in the early stage of drug development is indispensable and that the construction of a database based on toxicogenomics technology and its use with bioinformatics technology seemed to be the most effective strategy. However, for the size of the drug manufacturing concerns in our country, the economic load was too heavy, in contrast with the huge enterprises in Europe and the U.S., which can construct their own databases. In this situation, the Ministry of Health, Labour and Welfare, the National Institute of Health Sciences (NIHS), and a working group of the Japan Pharmaceutical Manufacturers Association began to draft a research project in which both the government and private companies joined. Preliminary examination was carried out mainly in the Cellular and Molecular Toxicology Division, Biological Safety Research Center of the NIHS in 2000 to 2001 [7], and the project "Construction of a forecasting system for drug safety based on the toxicogenomics technique and related basic studies" for the five years from March 2002 to March 2007 was started. Half of the entire budget,

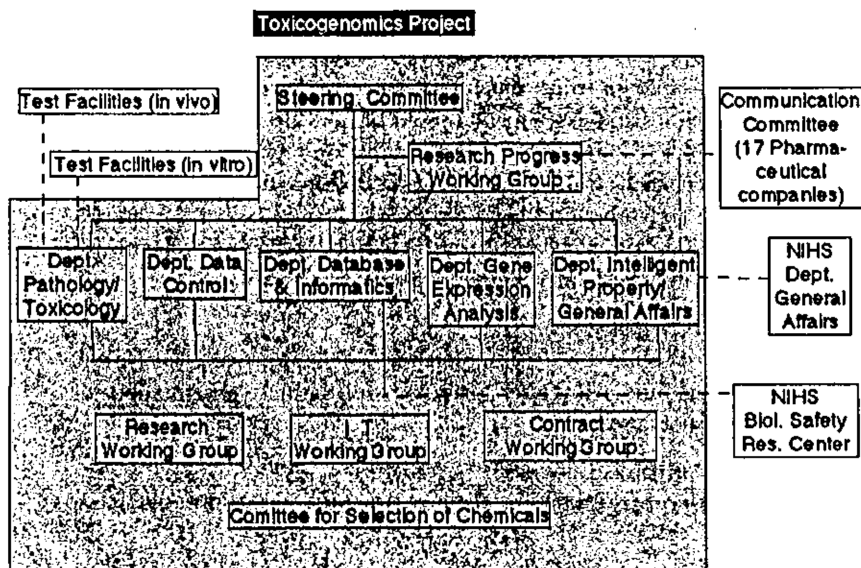

Fig. 26.3 Schematic map of the Toxicogenomics Project. The shadowed area is within the project.

about five billion yen, comes from the national budget, and the remaining half is contributed by the 17 companies that participated in the project within the Japan Pharmaceutical Manufacturers Association. Hitherto in Japan, cooperative research projects between national and private organizations have not been very active, especially in the field of pharmaceutical science. This project thus attracts attention from various quarters as a model case for the near future.

The schematic map of the project is shown in Figure 26.3. The project leader is the Director General of the NIHS, and researchers from the NIHS as well as those from the 17 drug companies participate in each department. The final goal of the project is to construct a large-scale toxicology database of chemicals and to develop a system for forecasting the toxicity of new chemicals. Due to the nature of this project, it is intended that distribution of the contents of the database and forecasting system will be limited to the project members until three years after the end of the project, and thereafter it will be opened to the public. Therefore, we would like to ask the readers' understanding that a detailed description of actual data is not possible in this paper.

26.3.2
Contents of the Project

To begin with, about 150 chemical compounds are selected, and the following are examined for each.

1) In-vivo tests using the rat
   The species selected for analysis was the rat, which is very frequently used in pre-clinical examinations and for which much toxicological information has been accumulated. The facilities and experimental protocol coincide with the GLP. The test consists of a single-administration test (multiple time points with multiple dose levels) and a repeated-administration test (multiple length with various dose levels), and data on body weight, general symptoms, histopathological examination of liver and kidney, and blood biochemistry are obtained from each animal. Gene expression in liver and kidney is comprehensively analyzed.

2) In-vitro tests using rat hepatocytes
   For rat liver primary cultured cells, comprehensive gene expression analysis is carried out at various time points after treatment with various concentrations of each of the 150 compounds.

3) In-vitro tests using human hepatocytes
   Using human liver primary cultured cells, experiments equivalent to the above are carried out.

Before starting the project, preliminary examinations (especially for the acquisition of gene expression data with absolute values) were carried out in the Cellular and Molecular Toxicology Division, Biological Safety Research Center, NIHS. In the course of examination, various problems became evident when various microarray methods were compared. For comprehensive analysis of gene expression using a microarray, two commercial methods were evaluated, i.e., the Affymetrix system and the Stanford system. Although the Stanford system is excellent in view of the cost, the Affymetrix system is superior in specificity and quantification. It was felt that meaningful toxicological changes could not be detected by a simple semiquantitative comparison like the two-colour method, since the purpose is expression analysis in the organ that is damaged by a chemical substance. Furthermore, it is necessary to measure the absolute value of gene expression so as to evaluate the time course and dose–response to the chemicals.

With the Affymetrix system, some quantification of the expression level based on the total amount of mRNA in the sample is assured. However, when a damaged organ is compared with a normal one, the observed change in gene expression can be masked by various noise, such as a change in the total gene expression level. In this regard, the desired measure is the absolute content of mRNA per cell. Based on this requirement, a system that determines the gene expression quantity per cell was developed utilizing the spike included in the Affymetrix GeneChip (Kanno et al., submitted for publication). With this system, it became possible for virtually all genes on the GeneChip to be detected as a change or no change in the absolute contents. In the Toxicogenomics Project, this system has been adopted and the data are now being accumulated.

26.3.3
Advantage and Originality of the Project

A number of toxicogenomics projects with various scales and objectives, national or private, are now being performed worldwide. Here, the advantages and originality of our project are listed:

1) The project is specialized for drug development
The database constructed in this project is expected to estimate the possibility of potential side effects in the earliest stage of drug development, to enable selection of the candidate with the smallest risk in clinical trials. To achieve this purpose, the project employs a unique strategy. That is, the drug selection committee selects a "drug for which a clinical trial was terminated or whose marketing was ceased because human toxicity emerged, even though the potential for toxicity was undetected or neglected during preclinical tests", mainly from the 17 companies participating in the project. At present, selection is done with the aim of having a group of about 50 such drugs, which will be subjected to the tests described above.

2) Various types of high-quality data are linked to the gene expression database
It is sometimes seen in a gene expression database that some data are from different sources or some or all data lack related biochemical or histopathological information. Our project aims at the construction of a database with a perfect dataset. That is, the gene expression data from each individual animal is always linked to its biochemical data, pathological evaluation, images of the histopathology and their interpretation, chemical structure of administered drug, and relevant literature. The database includes not only the basis of the toxicity-forecasting system but also functions as a standard large-scale archive of hepato- and nephrotoxicity.

3) The database contains absolute values for gene expression levels
As described above, gene expression data accumulated in this project are in the form of absolute values per cell. Circadian variations in the expression of each gene can be detected in the solvent-control population, meaning that the drug effect becomes observable. Furthermore, this may enable us to analyze the pharmacological action, i.e., pharmacogenomics, by analyzing the gene expression pattern at lower doses, at which toxicity does not emerge, since most of the chemicals tested are intended as medicines.
Although this database is unique, comparison with existing databases or with the usual nonquantitative analysis in the laboratory is also possible. According to this principle, it is easy to convert the absolute expression values into relative values, and thus qualitative comparison with other databases can be carried out.

4) Interspecies bridging is considered
Development of the system is aimed at the prediction of human toxicity. Since human experimenttation is impossible in any event, a laboratory animal must be used and subsequently the problem of species differences cannot be avoided. In this project, a full set of experiments, i.e., in-vivo rat tests, tests on rat primary

cultured hepatocytes, and tests on human primary cultured hepatocytes will be completed for all chemical compounds with a controlled protocol. In this system, extrapolating from rat to human may be possible through comparing the gene expression profiles of the cultured cells. Of course, we realize that many problems remain, e.g., it may be possible that differences between in-vitro and in-vivo tests is stronger than the species differences. We are now optimistic concerning this issue and hope that data accumulation will allow other outcomes, such as identification of a specific group of genes that enables interpretation and bridging the species.

26.4
Future Perspectives and Conclusions

When this chapter was written, in the summer of 2003, the project had just started on animal experiments. At present, about 50 standard drugs with typical hepatic toxicities have been listed, and for some of them, including acetaminophen, carbon tetrachloride, and ionized, data acquisition has already been completed. Although practical evaluation of the project is a matter for the future, it should be useful to describe the future view based on the present situation.

As recent advances in the 'omics' technology are rapid, it sometimes happens that an expensive facility becomes out of date only half a year after its inauguration. This means that the quality and quantity of data are being vigorously improved, but it also means that standardization of methodology is never achieved. Especially in the field of toxicology, data validation between each facility is important. It would not be extreme to argue that integrating databases without methodological standardization is useless. The technique and strategy adopted by our project have significance in that they supply the platform for standardization of the toxicogenomics technology. In the present situation, there is still an objection as to whether this approach can be used in the field of regulation. However, once standardization is achieved, and the reliability and predictability of the data become superior to the toxicological index produced by the old approach, it should lead to large innovations in the regulatory scientific field.

At present, changes in the expression of various interesting genes are being found to participate in the toxic response. The problem facing us is that considerable numbers of such genes on a GeneChip are functionally unknown ESTs, and that, even for the known genes, their functions and functional partners are little understood. It might be possible to construct a forecasting system in ignorance of the physiological, pharmacological, or toxicological facts, but this does not seem promising. When the future of this technology is considered, it is important to bring the significance of gene expression changes into the system, by active collection of the ever-progressing information of the genome. In this connection, it is necessary to periodically update and improve the database during and after the project, to create a perfect database. It will be also fruitful to connect it with other types of databases, such as proteomics, metabolomics, etc., databases, creating a giant network of toxicology databases. In

fact, another project of toxicogenomics has been just started with the initiative of the Cellular and Molecular Toxicology Division, Biological Safety Research Center, NIHS for the three years from 2003 to 2006. In this project, about 100 compounds, most of which are industrial and environmental chemicals, are to be tested for their effects on the gene expression profile of the mouse. The purpose of this project is also to create a database of chemical hazards based on absolute gene expression values per cell type. The greatest advantage of this project is that highly informative data for analyzing the mechanism of toxicity will be accumulated, since mouse genome information is nearly complete and various gene-modified mice are available. This would be a dream, but we hope that the integrated system will enable elucidation of the mechanisms of toxicity and prediction of human toxicity, in spite of species or individual differences, for any type of compound.

After this database and toxicity-prediction system are complete, it will be widely utilized in the basic technology of drug development. This will accelerate the early stages of preclinical trials by enabling wise choice of drug candidates and will subsequently decrease the rate of failure in clinical trials. This, we hope, will ultimately contribute to human welfare through the rapid supply of much safer and more useful drugs.

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Nitrofurazone-induced gene expressions in rat hepatocytes and their modification by *N*-acetylcysteine

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Abstract

The antibiotic nitrofurazone (NF) at a subtoxic dose has been shown to increase hepatocyte DNA synthesis with no preceding cell damage or necrosis. This was suppressed by concomitant administration of the antioxidant *N*-acetylcysteine (NAC), which suggests that free radical production is involved in the process. In this study, male F344 rats were given a single oral subtoxic dose of NF to investigate the changes in genes implicated in hepatocyte proliferation between 1 and 20 h postdose by real-time PCR. Some rats were also given NAC to examine the involvement of free radicals. There were transient and sequential increases in mRNA levels of c-myc and c-jun shortly after the administration, followed by tumor necrosis factor- α (TNF- α), transforming growth factor- α (TGF- α), c-Ha-ras, and cyclin E. The increases were blocked by concomitant administration of NAC. In contrast, there were no NF-specific increases in c-fos, hepatocyte growth factor, epidermal growth factor or cyclin D1 mRNAs. These results indicate that the induction of hepatocyte proliferation by NF is triggered by free radicals, with a pathway involving increases in c-jun, c-myc, TNF- α, TGF- α, c-Ha-ras, and cyclin E. The results also indicate that NF-induced proliferation resembles that of other mitogens.

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Keywords: Nitrofurazone; Rat; Hepatocyte; Gene expression; Proliferation; *N*-acetylcysteine; c-fos; c-myc; c-jun; c-Ha-ras; Tumor necrosis factor- α (TNF- α); Transforming growth factor- α (TGF- α); Hepatocyte growth factor (HGF); Epidermal growth factor (EGF); cyclin D1; cyclin E

Introduction

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone; NF), a broad-spectrum antibiotic, has been known to elicit toxic effects, including convulsive seizures, testicular degeneration, and degenerative arthropathy in rodents (Hagenäs et al., 1978; Kari et al., 1989). It has, in addition, been reported to induce necrotic changes in the liver and adrenal gland at high doses (Ito et al., 2004). In contrast, NF at low doses elicits hepatocyte proliferation without the preceding loss of hepatocytes, i.e. it acts as a mitogen (Ito et al., 2002). Indeed, in mice fed NF for 13 weeks, liver to body weight ratios were moderately increased (Kari et al., 1989), apparently without histopathological changes. The mitogenic effect is abrogated by concomitant administration of antioxidants *N*-acetylcysteine (NAC) or cyanidanol, indicating the involvement of free radical production (Ito et al., 2003). It is noteworthy that NF behaves as a hepato-

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toxicant at higher doses and as a mitogen at lower, subtoxic doses. Such is also the case with agents which are generally better known for hepatotoxicity such as thioacetamide (Mangipudy et al., 1995a, b) and carbon tetrachloride (CCl_4, Rao et al., 1997).

Proliferation of hepatocytes occurs very infrequently in normal adult rodents, the vast majority of cells being in the resting phase of the cell cycle (G_0). Hepatocytes, however, are equipped with mechanisms which allow them to quickly modulate the rate should the need arise. Two different types of proliferation are currently known, compensatory proliferation and mitogen-induced proliferation (Ledda-Columbano et al., 1993; Columbano and Shinozuka, 1996). Compensatory proliferation follows the loss of hepatocytes after partial hepatectomy or "chemical hepatectomy" by hepatotoxicants such as CCl_4. Mitogen-induced proliferation, in contrast, entails no overt damage or loss of hepatocytes. "Mitogens" include a broad spectrum of chemically unrelated substances such as lead nitrate (Columbano et al., 1983), cyproterone acetate (CPA, Roberts et al., 1995), cyclosporine (Masuhara et al., 1993), ethylene dibromide (EDB, Nachtomi and Farber, 1978), and hypolipidemic drugs (e.g. Wy-14,643, Rusyn et al., 2000), as well as a large number of stimuli. These include free radicals, decreases in protective enzymes, glutathione depletion, and sustained accumulation of normally low endogenous products (Iatropoulos and Williams, 1996). Although the two modes of proliferation share some of the events following the trigger, there are also differences in the series of changes in growth factors and early response genes. Compensatory proliferation involves increases in hepatocyte growth factor (HGF), transforming growth factor- α (TGF- α), epidermal growth factor (EGF), tumor necrosis factor-α (TNF- α), c-fos, c-myc, c-jun, and c-Ha-ras (Fausto and Mead, 1989; Fausto et al., 1995; Fausto, 1996), whereas some of these are unaltered in mitogen-induced proliferation (Coni et al., 1990, 1993; Masuhara et al., 1993; Goldsworthy et al., 1994; Shinozuka et al., 1994).

Much attention has recently been devoted to the mechanism involving free radicals. Free radicals at high concentrations inflict damage on cells, but at low doses are believed to act as a mediator of various signaling pathways within a cell (Remacle et al., 1995). The antioxidant NAC is widely used as an experimental tool in biological and pathological processes. Its mode of action is two-fold, as a free radical scavenger by directly reacting with them, and, additionally, it elevates intracellular glutathione content by providing a precursor (Zafarullah et al., 2003), thereby enhancing the cellular defense system. It has been used to counteract the effects of free radicals, and the mitogenic effect of NF has been prevented by its concomitant administration (Ito et al., 2003), suggesting the involvement of free radicals in the process.

This study examines the changes in gene expressions generally associated with hepatocyte proliferation in order to further characterize the nature of NF's mitogenic effect. The effect of NAC, which blocks free radicals and NF-induced proliferation, was concurrently examined for the modification of NF-induced genes in an attempt to elucidate to what extent free radicals contribute to the sequence of events following the use of NF.

Materials and methods

Animals

SPF male Fischer rats were purchased from Charles River Japan Inc. (Kanagawa, Japan), and acclimated until use in a room which was kept at 23 ±2 °C, with a relative humidity of 55 ±10%, ventilated 15 times/h, and lighted for 13 h (from 8:00 a.m. to 9:00 p.m.). The animals were given a commercially available diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The rats were 10–11 weeks old at the start of treatment. All animals received humane care throughout the experiment in compliance with the institutional guidelines for the care and use of laboratory animals.

Nitrofurazone and *N*-acetylcysteine administration and sample collection

NF (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was suspended at a concentration of 16 mg/mL in a 0.5% methylcellulose solution. NAC (Wako Pure Chemical Industries, Ltd.) was dissolved in saline at a concentration of 20 mg/mL.

Rats were given a single dose of NF at 80 mg/kg (5 mL/kg) by gavage using a stomach tube. Five animals were sacrificed at 1, 2, 5, 8, 12, 16, and 20 h postdose, respectively (NF rats). In addition, five rats each were given an intraperitoneal injection of NAC at 50 mg/kg (2.5 mL/kg) 30 min before and, where applicable, 5 h after NF administration, and sacrificed as above (NAC+NF rats). Five rats given a 0.5% methylcellulose solution alone served as controls at each time point.

Rats were sacrificed by exsanguination under ether anesthesia. The liver was excised for total RNA extraction.

RNA extraction and reverse transcription

The liver was homogenized in approximately 1 mL of ISOGEN (Nippon Gene, Tokyo, Japan) per 100 mg of tissue, and total RNA was extracted according to the manufacture's instructions. Total RNA was quantified

using a spectrophotometer set at a 260-nm wavelength. Contaminating DNA was removed from the RNA samples during a 15-min digestion at room temperature with DNase I, Amplification Grade (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA (0.8 μg) from each sample was converted to cDNA at a final volume of 20 μl using Super Script First-Strand Synthesis System for RT-PCR (Invitrogen Corporation) as instructed by the manufacturer. A thermal cycler GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA) was used. The cDNA was then diluted and used in subsequent PCR reactions

Real-time PCR

A length of the target gene was amplified in a mixture of water, cDNA, primer pair, and SYBR ® Green PCR Master Mix (Applied Biosystems) using an ABI Prism 7700 or 7900 HT (Applied Biosystems). The final concentration of the primers was 200 nM each. The number of cycles was between 40 and 48, and the annealing temperature was between 58 and 60 °C. The sequences of the primers used for each of the genes analyzed are described in Table 1. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Taqman ® Rodent GAPDH Control Reagents (Applied Biosystems) with a product size of 177 bp. Electrophoretic analysis of expected product sizes was performed for all primer sets prior to real-time RT-PCR to confirm the fidelity of the reaction. The standard curves were generated using a dilution series of corresponding purified PCR products. The data were processed using a Sequence Detection System (Applied Biosystems). A housekeeping gene, GAPDH, was used for normalizing the amount of each sample. The data were analyzed statistically with the Student's *t*-test to compare treated groups with the control, expressed as mean ±standard error (SE), and illustrated as a ratio to the control.

Results and discussion

Fig. 1a illustrates changes in c-jun mRNA levels between 1 and 8 h postdose in control, NF, and NAC+NF rats. The expression of c-jun was increased at 1 and 2 h in NF rats with a higher peak at 1 h. Similarly, c-myc was increased significantly in NF rats during both hours, but experienced a peak at 2 h (Fig. 1b). No increase in c-fos was specific to NF rats (data not shown). Another protooncogene, c-Ha-ras, was increased in NF rats at a later phase, between 8 and 12 h (Fig. 1c). The increases in protooncogenes c-jun, c-myc, and c-Ha-ras were partially or completely abolished in NAC+NF rats, indicating the NAC inhibited the effect of NF in enhancing the expression of these genes. TNF- α in NF rats was increased from 2 to 8 h, but decreased to the control level at 12 h (Fig. 1d). Changes in TGF- α between 5 and 16 h are shown in Fig. 1e. The expression was highest at 8 h and higher, but statistically insignificant at 12 h. Cyclin E was slightly increased in NF rats at 16 h, and further at 20 h (Fig. 1f). Again, increases in these genes after NF administration were suppressed by concomitant administration of NAC. An apparent increase in cyclin E was seen at 8 h in NF rats, but so was in NAC+NF rats; these are therefore likely to be biologically insignificant, together with the fact that there was no incorporation of BrdU at 12 h in a pilot study which should follow cyclin E expression. The increases, in sequential order, were in: c-jun, c-myc, TNF- α, TGF- α, c-Ha-ras, and cyclin E. There was no NF-specific increase in HGF, EGF or cyclin D1 expression (data not shown).

The results of the current study are strikingly similar to other mitogenic substances in the set of genes increased and those unaltered. Of the two proliferation modes, compensatory proliferation involves increases in growth factors including HGF, TGF- α, EGF, and TNF- α, and protooncogenes such as c-fos, c-myc, c-jun, and c-Ha-ras (Fausto and Mead, 1989; Fausto et al., 1995; Fausto, 1996). In contrast, although differences exist, mitogens are often associated with increases in c-

Table 1. Oligonucleotide sequences of PCR primers

Gene	Size (bp)	Forward	Reverse
c-myc	106	AGG AGA AAC GAG CTG AAG CGT A	CGG TGG CTT TTT TGA GGA TAA CT
c-jun	64	GAA AAC CTT GAA AGC GCA AAA C	CAC CTG TTC CCT GAG CAT GTT
c-fos	136	GAA GGG AAA GGA ATA AGA TGG CT	TTC AGT AGA TTG GCA ATC TCG G
c-Ha-ras	97	CTG GAC ATC TTA GAC ACA GCA GG	TGA TGG CAA ATA CAC AGA GGA AG
TNF- α	141	GTG ATC GGT CCC AAC AAG GA	TGC TTG GTG GTT TGC TAC GA
TGF- α	170	TGG GTC AAG GCC AAG TGT	TAT CAT CGG AAG CTA ACG GTG
HGF	83	CAA AAC AAG GTC TGG ACT CAC ATG	CGT CTG GCT CCC AGA AGA TAT G
EGF	218	TCA CTG GGA AAG ACT GCA AGA A	AGA TAC ACT GCA AGT GTG GCC C
Cyclin D1	86	CCG TCC ATG CGG AAG ATC	ATG GCC AGC GGG AAG AC
Cyclin E	149	GTG AAA AGC CAG GAT AGC AGT CA	CTG GGC GGT CTG ATT TTC C

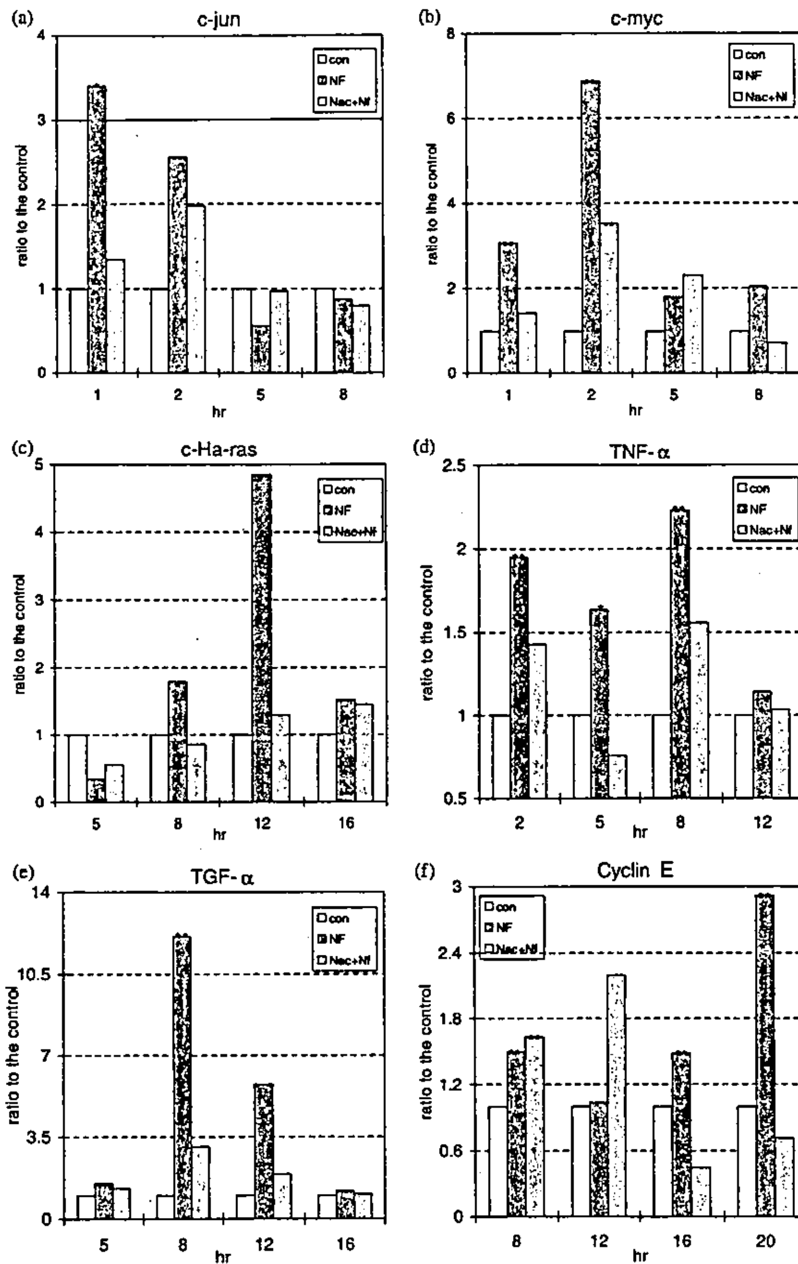

Fig. 1. (a) c-jun, (b) c-myc, (c) c-Ha-ras, (d) TNF- α, (e) TGF- α, and (f) cyclin E expression, respectively, following NF or NAC+NF treatment, and expressed as a ratio to the control. $^{*}P<0.05$ and $^{**}P<0.01$.

jun, c-myc, and TNF- α; increases in c-fos and HGF, however, are often absent (Coni et al., 1993; Goldsworthy et al., 1994; Masuhara et al., 1993; Shinozuka et al., 1994). In the present study, c-jun, c-myc, and TNF-α were increased in NF rats, and suppressed in NAC+NF rats. HGF and c-fos were not predictive of NF-induced hepatocyte proliferation. To what extent these NF-induced genes are involved in hepatocyte proliferation is unclear at this stage, but the fact that these genes were suppressed when hepatocyte proliferation was inhibited by NAC supports the notion that they are collectively responsible for NF-induced proliferation.

The majority of adult hepatocytes under normal circumstances are in the quiescent phase (G_0), but they re-enter the cell cycle in response to a proliferative stimulus. Hepatocytes undergo the process of "priming" during this step, which renders the cells capable of

responding to a further proliferative stimulus by going through a $G_0 \rightarrow G_1$ transition (Webber et al., 1994). Hepatocytes need both priming and growth stimuli for passing through the "restriction point" to enter the S phase. A one-third hepatectomy increases c-myc, but does not induce DNA synthesis (Webber et al., 1994). A single injection of HGF in normal rats induces no or only limited hepatocyte proliferation (Webber et al., 1994). In contrast, infusion of HGF or TGF- α to 30% hepatectomized rats resulted in significant increases in DNA synthesis (Webber et al., 1994). In the present study, increases in c-myc and possibly another proto-oncogene, c-jun, in NF-treated rats may have worked in the priming step, even though these genes may not have induced proliferation per se. In this context, it is of interest that the second administration of NF to rats induced a greater increase in DNA synthesis than the first dose (Ito et al., 2002). A larger portion of hepatocytes may have entered the cell cycle upon the second administration because of the priming by the first dose. A similar phenomenon is also known: in auto- and heteroprotection, a small dose of a chemical protects against a second lethal dose of the same or another compound, respectively (Chanda et al., 1995; Mangipudy et al., 1995a, b; Soni and Mehendale, 1998). It is considered that the smaller dose primes hepatocytes and enables them to proliferate more efficiently in an attempt to compensate for cell loss by necrosis.

The increase in TGF- α was unexpected since it is often unaltered by mitogens (Masuhara et al., 1993; Shinozuka et al., 1994). TGF- α is stimulated by HGF, and these are often expressed in concert. The expressions of both these genes are increased following partial hepatectomy, and, in contrast, are often suppressed in mitogen-induced proliferation. TGF- α is, however, thought to be more closely correlated with hepatocyte proliferation than HGF (Tomiya et al., 1998). TGF- α is also known to be directly up-regulated by TNF- α (Gallucci et al., 2000). There was no NF-specific increase in HGF in this study, whereas both TNF- α and TGF- α were increased. The increases in both these factors were seen at similar hours, but the elevation of TGF- α persisted longer than that of TNF- α. It is conceivable that TGF- α was induced by TNF- α, although other possibilities cannot be excluded.

The expression of c-Ha-ras was increased in NF rats between 8 and 12 h, later than other protooncogenes such as c-jun and c-myc. It is interesting that a similar pattern of increase in c-Ha-ras is seen with other mitogens including CPA and EDB, as well as in compensatory proliferation following partial hepatectomy and CCl_4 (Coni et al., 1990). The increase in c-Ha-ras expression is said to coincide with the increase in DNA synthesis and mitosis in these experiments. On the other hand, Ras activity functions late in G_1 phase and is required for passage through the restriction point, and entry into the S phase in growth factor-stimulated fibroblasts (Takuwa and Takuwa, 1997). No increase in DNA synthesis was seen in the hepatocytes of NF rats at 12 h postdose in a pilot study, the time when c-Ha-ras was increased. An increase in cyclin E followed that in c-Ha-ras. It is, therefore, more likely that c-Ha-ras is associated with the entry into the S phase in this experiment, rather than coinciding with DNA synthesis.

Cyclin D1, along with cyclin E, is said to be another critical protein in the G_1 phase, acting generally in sequence. Cyclin E is the major downstream target of cyclin D1. Contrary to our expectations, no increase in cyclin D1 was seen in parallel to, or slightly previous to, cyclin E expression in NF-treated rats. It is suggested that the roles of cyclin D1 can be bypassed and that cyclin E is more involved in directly activating the downstream events of G_1 progression or S phase entry (Roberts, 1999). Studies in other systems indicate that the activation of the cyclin E system is sufficient to trigger cell cycle progression in the absence of cyclin D1 (Connell-Crowley et al., 1998). Also compatible with this idea is that the replacement of cyclin D1 with cyclin E can completely rescue the developmental defects observed in cyclin D1-deficient animals (Sherr and Roberts, 1999). These facts suggest that, although the possibility of posttranscriptional activation of cyclin D1 cannot be excluded, NF-induced hepatocyte proliferation may possibly be mediated by a pathway involving cyclin E, without cyclin D1 participation.

The hypothetical sequence of events associated with NF-induced hepatocyte proliferation can be summarized as in Fig. 2. It is accepted that free radicals within the cell's antioxidant capacity induce cell growth (Remacle et al., 1995). As mentioned above, the involvement of free radicals is implicated in NF-induced proliferation, which is suppressed by antioxidants. Various genes were increased following NF, but those that are inhibited by NAC are likely to play more critical roles in the process: c-jun, c-myc, TNF- α, TGF- α, c-Ha-ras, and cyclin E. Free radical generation during NF metabolism (Tatsumi et al., 1976; Peterson et al., 1979; Wolpert et al., 1973) is likely to rise almost immediately after administration, since the blood plasma concentration of NF peaks at 1 h postdose (Ito et al., 2002). Free radicals produced during NF metabolism may lead to increases in immediate early genes such as c-jun and c-myc, resulting in the priming of hepatocytes which have now acquired competence for cell growth. These events are followed by proliferative stimulation by TNF- α, or TGF- α, or both. The expression of c-Ha-ras, in concert with other genes including cyclin E, conceivably allow hepatocytes to pass through the restriction point and enter the S phase of the cell cycle, finally culminating in DNA synthesis. What is still unclear is the precise mechanism through which NAC suppresses NF-induced proliferation in

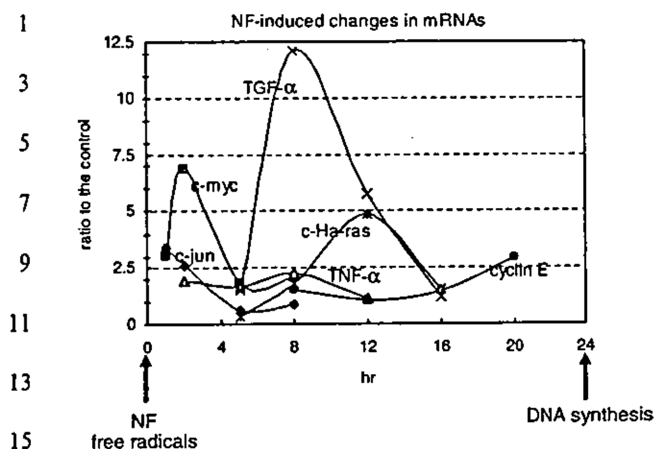

Fig. 2. Changes in the mRNAs of genes induced by NF. Free radicals produced during NF metabolism may lead to immediate early genes such as c-jun, c-myc, and possibly TNF- α. This results in the priming of hepatocytes which have now acquired competence for cell growth. These events are followed by proliferative stimulation by TNF- α, or TGF- α, or both. The expression of c-Ha-ras, in concert with other genes, including cyclin E, may conceivably allow hepatocytes to pass through the restriction point and enter the S phase of the cell cycle, finally culminating in DNA synthesis.

hepatocytes. It remains to be seen whether the inhibition of the above genes is an indirect result of free radical scavenging, eliminating the trigger itself and interrupting the cascade at the root, or a direct inhibitory effect on each step of the cascade by NAC, or even both, although the former is more likely considering the two-fold antioxidant action of NAC.

It is intriguing that a substantial portion of the changes associated with proliferation is shared among diverse, chemically unrelated substances acting as mitogens. Although the relative importance of each gene is yet to be elucidated, further studies, including changes at the protein level and activation of various transcription factors, should contribute to a better understanding of the process.

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Original Article

Microarray analysis of genes in fetal central nervous system after ethylnitrosourea administration

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Abstract

Ethylnitrosourea (ENU), a monofunctional alkylating agent, induces apoptosis and cell cycle arrest in neuroepithelial cells, neural stem cells in the fetal central nervous system (CNS). These effects occur immediately after the administration of ENU to pregnant animals resulting in fetal brain anomalies and long-term effects include brain tumors in the offspring. In the present study, changes in gene expression were investigated in the fetal CNS after ENU administration to pregnant rats using microarray to identify the genes involved in the injury and recovery of the fetal CNS. The up-regulation of 21 genes in injury and 15 genes in recovery phases and down-regulation of 5 genes in injury and 3 genes in recovery phases were identified. The genes up-regulated in the injury phase contained p53-target genes that mediate apoptosis and cell cycle arrest, and those in the recovery phase contained cell proliferation-promoting genes. The genes down-regulated in the injury phase contained cholesterol biosynthesis-related genes. In addition, there were some genes that have not been identified to be involved in the CNS injury and recovery. The results from the present study will provide a better understanding of the mechanisms of development, regeneration and carcinogenesis of the CNS as well as the mechanisms of ENU-induced fetal CNS injury and recovery.

Key words: apoptosis, cell cycle arrest, microarray, ethylnitrosourea, neuroepithelial cell, p53

Introduction

Neuroepithelial cells are neural stem cells that exist in the fetal central nervous system (CNS). They are multipotent stem cells that can self-renew and differentiate into cells of both the neuronal and glial lineage (Yoshikawa, 2000). Ethylnitrosourea (ENU), a monofunctional alkylating agent, selectively induces brain tumors in offspring when administered to pregnant animals, and this experimental model has been extensively used to investigate the pathogenesis of CNS tumors (Koestner, 1990; Jang et al., 2004). ENU induces apoptosis and cell cycle arrest in neuroepithelial cells in the fetal CNS immediately after its administration to pregnant dams (Leonard et al., 2001; Katayama et al., 2001, 2005) and as a result, congenital brain anomalies can be induced in neonates (Katayama et al., 2000). It has been shown that neuroepithelial cell apoptosis and cell cycle arrest induced by ENU are mediated by the regulation of p53 and p53 target genes and efficiently inhibited in p53 knockout mice (Leonard et al., 2001; Katayama et al., 2002, 2005).

In the present study, changes in gene expression at injury and recovery phases were investigated in the fetal CNS after ENU administration to pregnant rats on day 13 of gestation using microarray to identify the genes involved in the injury and recovery of fetal CNS.

Materials and Methods

All procedures were performed in accordance with the protocol approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Animals