Review

Phenotypic reversion in analbuminemic rats due to an altered splicing mechanism

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(Contributed by Takashi Sugimura, M.I.A.)

Abstract: Serum albumin is regarded as an important and indispensable protein, but analbuminemic rats established by Sumi Nagase in 1977 seem to exhibit few symptoms in spite of an almost total lack of albumin in the serum. The albumin gene of analbuminemic rats was found to have a seven-base-pair deletion in an intron, close to exon-intron junction, resulting in the formation of non-functional mRNA in hepatocytes. Immunostaining for albumin was negative in young analbuminemic rat hepatocytes, but a significant number of immunoreactive hepatocytes were observed in aged rats. The incidence of immunoreactive hepatocytes increased with aging. Surprisingly, many immunoreactive hepatocytes were observed after hepatocarcinogen treatment sometimes in large clusters. Albumin transcripts in analbuminemic rat liver after treatment with carcinogen, showed an altered pattern of exon-skipping. The altered albumin molecules thus synthesized accumulated in cellular organelles. Analbuminemic rats exhibited a high sensitivity in various organs to different types of carcinogens. Further challenges remain regarding the biology of analbuminemic rats.

Keywords: analbuminemic rat, exon skipping, alternative splicing, aging, carcinogenesis, albumin gene

History of the start of analbuminemic rat studies

In 1977 an analbuminemic rat was found by Sumi Nagase among a stock of the Sprague-Dawley strain in the Clinical Chemistry Laboratory of the Sasaki Institute. She was investigating standard values for serum parameters and one day was astonished to find a total lack of serum albumin in a rat, as analysed by electrophoresis. She immediately established a strain of analbuminemic rats by genetic crossing and discovered that the analbuminemia was an autosomal recessive trait. The body weight, longevity, fertility, litter size, and lactation of the strain were almost completely normal and the rats appeared quite healthy. Nagase subsequently published an excellent review on the genetic background and the biological characteristics of analbuminemic rats in Science.1

We had the good fortune to learn of this unique rat strain from Nagase, who telephoned one of the authors (TS) to convey the exciting news about her discovery of the mutant rat. She complained that nobody believed her surprising discovery but Nagase brought an electrophoregram of the serum proteins of an analbuminemic rat to our laboratory and, as shown in Fig. 1, the absolute lack of albumin band was clear. We therefore launched a collaborative study to elucidate the underlying molecular mechanisms.

Elucidation of the molecular mechanism responsible for the total absence of albumin

The first task was to isolate the albumin gene from analbuminemic rats. One of authors, HE, tried to sequence the albumin gene and the adjacent regulatory region. Then, while attending a Gordon Research Conference on Cancer, New London, Con-
necticut, in the summer of 1979, TS visited Alfred Knudson at his Institute, Fox Chase, Philadelphia and took the opportunity to discuss this rat strain with Shirley M. Tilghman, who had just isolated a cDNA clone for rat albumin. Knudson arranged for her to provide us with the clone and this greatly accelerated our progress.

It soon became clear that the seven bases were missing in one of the 14 introns, intron (H-I) of the gene in the analbuminemic rat, as shown in Fig. 2. The loss started from the fifth base from the intron (H-I) junction. Abundant immature albumin mRNA was found to accumulate in the nuclei of the hepatocytes of the mutant animal, but mature and functional albumin mRNA was absent in the cytoplasm.

Synthesis of α-fetoprotein and albumin and albumin mRNA

It is well known that α-fetoprotein is synthesized in the embryonic liver and replaced by albumin 2-3 weeks after the birth. As expected, in alb+/+ control rats, serum α-fetoprotein concentrations reached a maximum just after birth, about 10 mg/ml, and gradually decreased to almost undetectable level at 4 weeks thereafter, with concomitant increase of serum albumin concentration. In analbuminemic rats (alb−/−), changes of serum α-fetoprotein concentrations mirrored those in normal rats, but the serum albumin concentration did not increase after birth at all, suggesting that shut-off of α-fetoprotein gene transcription took place despite the lack of albumin increase (Fig. 3a). Consistent with these findings, immature mRNA for albumin began to appear from 1 week after birth and increased further during the following a few weeks as in the normal rat (alb+/+) liver (Fig. 3b). This phenomenon indicated that the switch-on and -off mechanisms for α-fetoprotein and albumin occurred at the gene-transcription level.

Age-dependent appearance of protein reactive with anti-albumin antibody in serum and liver

When formalin-fixed sections of the livers of analbuminemic rats were subjected to immunostaining using anti-albumin antibody, the entire liver was found to lack staining. However, mysteriously the presence of cells immunoreactive to anti-albumin antibody (immunoreactive cells) was noted at a rate of one per several microscopic fields under 100-times magnification. Interestingly, very minute amounts of a serum protein reactive with anti-albumin antibody were also present in alb−/− rats, and increased with age, as shown with open circles in Fig. 4. Indeed, clusters of several cells that were immunoreactive with the anti-albumin antibody were readily detectable in aged alb−/− rats, and their incidence increased over time. We suspected phenotypic reversion of albumin-negative cells to albumin-positive cells were not able to prove this at the time. Consistent with this idea, serum concentration of protein reactive with anti-albumin antibody increased in carcinogen-treated rats more significantly than in untreated rats, but the effect of carcinogen treatment on increment was about 2 fold over that in untreated rats (Fig. 4).

Dramatic increase in immunoreactive cells in the livers of alb−/− rats with hepatocarcinogen administration

We were further surprised to observe an increase in the number and size of clusters of immunoreactive cells in the livers of alb−/− rats after feeding a diet containing a typical hepatocarcinogen, 3’-methyl-4-dimethylaminoazobenzene or 2-acetylaminofluorene (Fig. 5a and b). Very large foci of immunoreactive cells were then observed, along with numerous isolated immunoreactive cells and small foci. As described previously, there was a concomitant increase in protein immunoreactive with anti-albumin antibody in the serum, but this was far less prominent.
than the increase in immunoreactive cells (Fig. 4). In other words, there was an apparent discrepancy between the increase in reversion of cells (more than 1000 fold) and protein in the serum, (limited to about 5 fold).

Our interest was further stimulated by the finding that a carcinogen like N,N-diethylnitrosamine, which ethylates DNA bases but does not form bulky DNA base adducts, did not cause significant increase in immunoreactive cells. As revealed subsequently, the exon skipping phenomenon occurring in the livers of alb−/− rats may be related to the mode of action of carcinogens that form bulky DNA-base adducts.

Mechanism of expression of the immunoreactive protein

How was it possible to convert hepatocytes missing seven bases in an intron of albumin gene to cells able to produce albumin molecules? To answer this question, we isolated albumin mRNA from the foci of immunoreactive cells. Overcoming various daunting problems, we eventually succeeded in identifying albumin mRNA that could be translated into albumin protein with a smaller molecular weight than standard albumin. A summary of the results is schematically represented in Fig. 6. The amounts of cytoplasmic albumin mRNA in the livers of young alb−/+ rats not treated with carcinogen was only trace compared to the levels in alb+/+ rats, but most cytoplasmic albumin mRNA was missing exon H and had been formed by exon skipping between exons G and I.

With the hepatocarcinogen administration to alb−/− rats, cytoplasmic albumin mRNA missing both exons G and H or exons H and I increased, indicating that splicing occurred between exons F and I (G and H exon skipping) and between exons G and J (H and I exon skipping). Albumin’ formed from the mRNAs produced by these alternative exon skipping would be expected to have different structures of the C-terminal portion and thus differences in intracellular localization. Albumin accumulation and altered subcellular localization was observed by immunoelectron microscopy in the liver and altered subcellular localization was further confirmed by expression of albumin mRNAs produced by different
Fig. 3. Albumin transcription and serum AFP concentrations.

a. Time dependent change of serum AFP (●) and serum albumin (○) concentration after birth in alb+/+ and alb−/− rats.

b. Time dependent change of serum AFP concentration (○) and amount of albumin gene transcript (●) in the nuclei of the liver of alb+/+ and alb−/− rats.

The serum AFP concentration was measured by a single radial immunodiffusion method using a specific antibody against AFP. RNA was extracted from rat liver nuclei and quantitated by a method involving RNA-cDNA hybridization followed by S1 nuclease digestion (Modified from Cancer Res. 42, 306–308 (1982)).

Examples of exon skipping in COS cells followed by immunofluorescence microscopy. Albumin produced by mRNA derived from H and I exon skipping was found to be distributed in the Golgi apparatus, endoplasmic reticulum, and secretory vesicles, although normal albumin is secreted quickly from cells. The mechanism of the accumulation was assumed to be impaired excretion of short sized albumin molecules by COS cells and by hepatocytes from carcinogen treated animals. Such an impairment might also occur with other proteins produced by altered splicing fidelity and/or specificity during carcinogenesis and may be one typical epigenetic change occurring during neoplasia.

It is now well documented that very many factors are involved in splicing, including nucleases, specific binding proteins, kinases, and phosphatases. This means that the number of genes targeted by carcinogens producing bulky DNA adducts is enormous, and the yield of mutated protein species in relation to splicing should account for a high yield of phenotypic reversion.

Dramatic change in susceptibility to chemical carcinogens

We also investigated the sensitivity of alb−/− rats to carcinogens. Remarkably high sensitivity to N-butyl-N-(4-hydroxybutyl) nitrosamine in-
Fig. 4. Change in anti-albumin antibody-reacting protein concentrations in serum of analbuminemic rats with age and in response to a carcinogen. Carcinogen treatment started at 4 weeks after birth. Serum anti-albumin was measured by a single radial immunodiffusion method using a polyclonal antiserum against rat serum albumin. Open circles indicate anti-albumin antibody-reacting protein concentrations in the serum of analbuminemic rats without carcinogen treatment and solid circles are for rats fed on 0.06% 3'-methyl-4-dimethlaminoazobenzene in basal CE-2 pellet diet (adopted with permission from Biochem. Biophys. Res. Commun. 106, 863–870 (1982)).

Fig. 5. Appearance of albumin-immunoreactive cells in a carcinogen-treated analbuminemic rat. a. A large cluster of immunoreactive cells after administration of 0.06% 3'-methyl-4-dimethlaminoazobenzene in basal CE-2 pellet diet for 20 weeks to analbuminemic rats. Albumin-positive cells were detected by immunostaining with a polyclonal antiserum against rat serum albumin. b. Time-dependent change in number of albumin-positive hepatocytes during hepatocarcinogen treatment. •—•, 3,4-A-methyl-4-dimethlaminoazobenzene-treated rats; ■—■, 2-acetylaminofluorene-treated rats; △—△, N, N-diethylnitrosamine-treated rats; ▴—▴, control rats.

Induction of urinary bladder cancer (Fig. 7) was a very early finding. Several carcinogenesis experiments were subsequently conducted and the results are summarized in Table 1. In addition to N-butyl-N-(4-hydroxybutyl) nitrosamine-induced bladder carcinogenesis, alb−/− rats showed remarkably elevated sensitivity to kidney carcinogenesis induced by dimethylnitrosamine, gastric carcinogenesis induced by N-methyl-N′-nitro-N-nitrosoguanidine, breast carcinogenesis induced by
Fig. 6. Schematic representation of aberrantly spliced albumin mRNA. Albumin gene consists of 15 exons, Z,A,B,–,N, but for simplicity, only part of the mRNA structure is presented in this figure.

Fig. 7. Gross appearance of urinary bladder tumors in analbuminemic rats. Tumors were induced by administration of 0.045 to 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine in drinking water for 20 weeks. Left half, bladders of alb−/− rats. Right half, bladders of alb+/+ rats (adopted with permission from Br. J. Cancer 45, 474–476 (1982)).
Table 1. Sensitivity of analbuminemic rats to various chemical carcinogens

| Carcinogen | Target organ | Sex | Incidence of tumor |
|------------|--------------|-----|--------------------|
| BBN        | Bladder      | male| NAR: 16/16; SD: 3/18 |
| 3'-Me DAB  | Liver        | male| NAR: 25/32; SD: 28/30 |
|            |              | female| NAR: 23/35; SD: 16/29 |
| DMN        | Kidney       | male| NAR: 38/50; SD: 23/62 |
| MNNG       | Stomach      | male| NAR: 12/17; SD: 8/21 |
| DMBA       | Breast       | female| NAR: 7/20; SD: 18/25 |
| AM         | Intestine    | male| NAR: 23/23; SD: 21/29 |
| 3MC        | Skin         | male| NAR: 24/24; SD: 19/20 |
| Subcutis   |              | male| NAR: 7/8; SD: 4/12 |

BBN, N-butyl-N-(4-hydroxybutyl) nitrosamine; 3'-MeDAB, 3'-methyl-4-dimethlaminoazobenzene; DMN, N,N'-dimethylnitrosamine; MNNG, N-methyl-N-nitro-N-nitrosoguanidine; DMBA, 7,12-dimethylbenz[a]anthracene; AM, azoxymethane; 3MC, 3-methylcholanthrene; NAR, analbuminemic rats; SD, Sprague Dawley rats. Incidence of tumor is expressed as number of animal with tumor over total number of animal used in the experiment (adopted from Jpn. J. Cancer Res. (1988) 79, 775-784).

7,12-dimethylbenz[a]anthracene, colon carcinogenesis induced by azoxymethane, and subcutaneous carcinogenesis induced by 3-methylcholanthrene, as compared to their counterpart alb+/+ Sprague-Dawley rats. These differences in sensitivity may be explained either by altered metabolic activation and inactivation of carcinogens in the liver and other organs, or by transportation of carcinogens and their metabolites through binding to serum albumin.

Implications of alternative splicing and exon skipping for carcinogenesis

Alterations in the primary structure of genes, copy numbers of genes, and transcriptional regulatory genes involved in carcinogenesis have been well documented as mechanisms contributing to carcinogenesis. In addition to such alterations, structural and functional changes of proteins through different patterns of alternative splicing of gene transcript have attracted the attention of cancer biologists. This may be a new and important paradigm for carcinogenic pathways. Typical examples of functional alterations of key proteins due to changes in splicing patterns of mRNAs involve the epidermal growth factor receptor, fibronectin, CD44, and MDM2. These findings imply that alterations in mechanisms controlling post-transcriptional maturation to formation of functional mRNA may play important roles in carcinogenesis. This is a novel concept of intertwined genetic and epigenetic mechanisms underlying carcinogenesis.

Returning now to analbuminemic rats, the genetic defect is a 7-base-pair deletion in an intron of the serum albumin gene that causes a functional inactivation of 5'-end of an intron in mRNA splicing. With aging, and, more strikingly, upon hepatocarcinogen treatment, the pattern of exon skipping changes dramatically. It is also interesting that the efficiency of ‘apparent reversion’ varies with the mode of action of the carcinogen. It is well known that enzyme altered foci with changed expression of glutathione-S transferase, for example, are induced in the liver by hepatocarcinogens and their distribution is somehow similar to that of the appearance of albumin-immunoreactive cells. Therefore, it is of interest to elucidate whether there might be common mechanisms of induction.

Future perspectives

The background level of serum albumin reactive with anti-albumin antibody in the analbuminemic rat is very low, and thus it provides an ideal model for gene therapy. The physiological changes that occur to maintain protein osmotic pressure in serum are of interest and hyperlipidemia is observed in the analbuminemic rat. About 40 human cases of analbuminemia have also been reported, most of them not exhibiting clinical manifestations other than hypercholesterolemia, especially LDL-cholesterolemia. Lipodystrophy has furthermore been described in some human analbuminemic subjects. Serum albumin is normally the most abundant serum protein and is thought to be exclusively produced in the liver. Because the absence of serum albumin background, it is possible to ask if any other organ or tissue synthesizes serum albumin formed by exon skipping under physiological or pathophysiological conditions in the Nagase analbuminemic rat.

The urinary bladder, kidney, colon and stomach of analbuminemic rats show extremely high sensitivity to carcinogens that specifically induce cancers in these organs, but the mechanisms underlying this hypersensitivity have yet to be elucidated. Since
serum albumin is one of most important carriers of hydrophobic compounds, the pharmacokinetics and pharmacodynamics of carcinogens could clearly be altered by its absence. Hyperlipidemia has recently been found to be one of the most crucial characteristics determining the incidence of colon cancer in min mice which have a mutation in the \textit{APC} gene and are a model of familial polyposis. Colon carcinogenesis can be suppressed by lowering hyperlipidemia in this animal model of genetic colon cancer.\textsuperscript{19} Hyperlipidemia or hypercholesterolemia in analbuminemic rats may thus be related to their hypersensitivity to carcinogens.

Some genetic defects are caused by mutations at splice junctions, and alternative protein products may be deleterious, especially in non-cycling cells, such as neurons. Supplying wild-type protein by gene transfer appears inadequate to overcome problems, and release from the original defect may be required to restore wild-type protein synthesis. One intriguing possibility is that suppression of the impaired splicing caused by the mutation might be possible by introducing altered splicing machinery using this analbuminemic rat as a model.\textsuperscript{20} Modifications of DNA bases by carcinogens has attracted a great deal of attention from cancer researchers but carcinogens also modify proteins. Recently, posttranslational modification of many proteins, especially nuclear proteins, has been found to play important roles in regulating cellular functions. Once modification of a critical protein triggers change in gene regulation, phenotypic alteration of cell might not necessarily be transient. Proteomic analyses of immunoreactive cells are clearly warranted.

Elucidation of the \textit{in vivo} mechanisms responsible for the lack of serum albumin and physiological compensation for the lack of this normally most abundant serum protein was carried out more than two decades ago. However, the recent tremendous advances made in gene, transcript and protein analysis technology, should now make it possible to elucidate the molecular mechanisms more precisely and facilitate practical application of our understanding. Knocking out albumin gene in mice having various genetic backgrounds must be of most interesting, but no such information is available at present.

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