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STUDIES ON THE CORRELATION BETWEEN GLUCURONIC ACID METABOLISM AND BETA-GULUCURONIDASE ACTIVITY IN CANCER HOSTS

Takeo Wada, Hiromichi Ohara, Yoshiro Ishii, Atsushi Noto,
Akira Kihara, and Hideki Noguchi

Department of Medicine
Sapporo Medical College and Hospital

Among metabolic derangements commonly found in patients with malignant diseases, the development of cachexia in the advanced stages presents a formidable problem. In spite of various biochemical studies (1, 2), no specific metabolic change, in the strictest sense, has been ascribed to cancer cachexia. On the contrary, metabolic changes found so far in carcinoma patients are only quantitative, but not qualitative, when compared with the metabolism in normal subjects or in patients with non-malignant diseases. Nevertheless, the search for the mechanism of cachexia formation still appears important, inasmuch as there should be functional disorders comparable to the metabolic derangements, if only quantitative, on the side of the cancer hosts. Also, attention must be called to the source of the dysfunction in the detoxication processes, which should normally be able to cope with the metabolic derangements toxic to the hosts.

In starting a series of biochemical studies on the detoxication by glucuronic acid, which is widely accepted to play the main role in the

detoxication mechanisms in the body, the interrelation between the metabolism of glucuronic acid and the elevation of β-glucuronidase activity in malignant neoplasms was investigated in clinical and animal experiments.

Materials and Methods

1. Clinical Experiments

a. Determination of glucuronic acid (GA) concentration in serum: To 5 ml of distilled water was added 1 ml of the blood drawn by venepuncture to cause hemolysis. To the mixture were then added 2 ml of 0.3 N $Ba(OH)_2$ and 2 ml of 5% $ZnSO_4$ to remove protein. The filtrate was subjected to GA determination with Dische's carbazole method (3).

b. Assay of serum β-glucuronidase (βGase): Biosynthetic p-nitrophenyl-β-glucuronide (4) was used as the substrate in the enzyme assay. The substrate, 0.1 ml in 0.1 M concentration, was incubated with a mixture of 0.2 ml of the serum sample and 0.8 ml of acetate buffer, pH 4.0, at 37° C for 7 hours. After incubation, 1.0 ml of 0.1 N NaOH was added to the mixture to develop the color, and distilled water was added to adjust the final volume to 6 ml. Colorimetric measurement was performed at 400 mμ against the control containing no substrate. The activity of βGase was expressed as μg (unit) of liberated p-nitrophenol per 100 ml of serum per hour.

c. Determination of urinary glucuronides (GLN): Separation of free and N-glucuronide fraction (F+N-GLN) from O-glucuronide fraction (O-GLN) was carried out using anion exchange resin (IR-410) chromatography according to the method reported by Ishidate (5). The glucuronic

acid concentration in each fraction was determined with the carbazole method (3) as described above.

d. Measurement of βGase inhibitor in urine: The heat-in-acid potentiation method reported by Marsh (6) was adopted for the measurement of urinary excretion of βGase inhibitor after administrations of GA (500 ml of 5% GA solution, intravenous), of glucuronolactone (GAL, 5 g oral) or of glucaro-1, 4-lactone (G1:4L, 5 g oral). An aliquot of the 24 hour urine was treated at 100° C for 40 minutes after adjustment of pH to 2.0 - 2.2 with 3 N HCl. The pH was subsequently readjusted to 4.0 - 4.5 with NaOH. The heat-treated urine sample (0.1 ml) was added to a solution consisting of 0.1 ml of βGase enzyme solution (purified calf liver extract), 0.1 ml of 0.1 M substrate and 0.8 ml of acetate buffer, pH 4.7, and the mixture was incubated at 37° C for 4 hours. Colorimetric control was established by an addition of distilled water as the substitute for the urine sample. The βGase inhibiting activity was expressed in percentage of inhibition against the control.

2. Animal Experiments

a. Subcutaneous transplantation of Yoshida sarcoma: Wistar strain male albino rats previously given subcutaneous transplantations of Yoshida sarcoma were divided into four groups. Animals of group A (control group) received sarcoma transplantation alone, while animals of group B were treated with GA administrations starting from the 7th day prior to the transplantation. Animals of group C received GA administrations starting from the date of the transplantation, and those of group D recieved G1:4L administration starting from the date

of the transplantation. All animals were sacrificed on the 5th day after sarcoma transplantation.

b. Assay of tissue βGase: Aliquots of the liver, spleen, kidney, adrenal gland, and sarcoma tissues obtained from the rats were ground in acetate buffer, pH 4.0, to give concentrations (wet weight) of 0.4, 0.8, 2.0, 2.0, and 0.4%; respectively. After standing overnight at 4° C, the homogenates were centrifuged at 3,000 r.p.m. for 10 minutes, and the supernatant solutions were subjected to determination of βGase activity at pH 4.7. The βGase activities thus assayed were expressed as μg (units) of liberated p-nitrophenol per gram of tissue per hour.

c. Excretions of Gl:4L in urine and in bile: An external biliary fistula was established in a dog. Bile and urine samples were collected for four hours before and four hours after an oral administration of 3 g of Gl:4L. Both urine and bile samples thus obtained were potentiated by the heat-in-acid method as described above, and were subjected to the determination of the βGase inhibiting power.

3. In vitro Experiments

Inhibition of βGase activity by GA, GAL and Gl:4L : Each of the three substances in a dose of 0.2 mg was added into the purified calf liver extract (βGase preparation), and the direct inhibition of βGase activity was determined.

In the second step of the experiment, the same dose of either GA or GAL was added to a glucuronolactone-dehydrogenase preparation (7), which was the supernatant of a rat liver preparation extracted with

0.17 M KCl solution to make a concentration of 10%, and the mixture was incubated overnight at 37° C and at pH 6.5. It was subsequently boiled to destroy the enzymic activity, and the boiled supernatant was added to the mixture consisting of the βGase preparation, the substrate, and acetate buffer, pH 4.7. Finally the βGase inhibiting effect of this mixture was assayed.

In one experiment, an incubation mixture consisting of 0.5 ml of 50 mM GA or of 50 mM GAL, 1 ml of the above-mentioned rat liver preparation, and 3 ml of acetate buffer, pH 6.5, with or without an addition of 0.5 ml of 25 mM NAD, was subjected to incubation at 37° C for 3 hours prior to the heat-in-acid potentiation (7). One ml of the potentiated sample was added to a mixture consisting of the βGase preparation, the substrate and acetate buffer, pH 4.7, and the βGase inhibiting effect of the sample was assayed.

Results

1. GA Metabolism in Patients with Carcinoma

Blood GA concentrations in patients with carcinoma were slightly higher than the values in healthy controls. The GA level was highest in cases with advanced carcinoma (Table 1).

Urinary glucuronide (F+N-GLN & O-GLN) excretions were markedly higher in carcinoma patients than in the controls. This was especially true for F+N-GLN, the increase of which being statistically significant,

Table 1. Glucuronic Acid (GA) Level in Blood and Glucuronide (GLN) Content in Urine of Patient with Carcinoma and of Normal Control

	No.	GA in blood (mg/dl)	GLN in urine (mg/day)		
			Total-GLN	F+N-G	O-G
Control	10	6.4 ± 1.1	170.4 ± 76.3	65.4 ± 18.5	105.0 ± 41.6
Incipient and moderately advanced Ca.	9	6.7 ± 1.8	253.9 ± 111.8*	117.0 ± 97.7*	136.9 ± 74.2
Advanced Ca.	7	6.8 ± 1.3	458.6 ± 266.9*	286.3 ± 216.6*	172.3 ± 89.0

* Statistically significant differences from normal values are indicated: $P = < 0.01$

and the degree of the increase being in proportion to the advance of carcinoma. The increase of O-GLN excretion, on the other hand, was not statistically significant.

Serum βGase activity had a tendency to elevate beyond normal range in carcinoma patients (Fig. 1). The rate of the elevation was proportional to the advance of carcinoma rather than to the type of carcinoma.

The activities of βGase in sera and in various tissues of rats were compared between each other (Fig. 2). High activities were found in the liver and in the spleen, and a considerably high activity was also observed in sarcoma tissues obtained from rats having subcutaneously transplanted Yoshida sarcoma.

2. Effects of GA, GAL and of Gl:4L Administrations upon βGase Activity

The serum βGase activities were suppressed in all instances by administrations of GA, GAL, and Gl:4L (Fig. 3). The most remarkable suppression was found in the case of Gl:4L administration, the

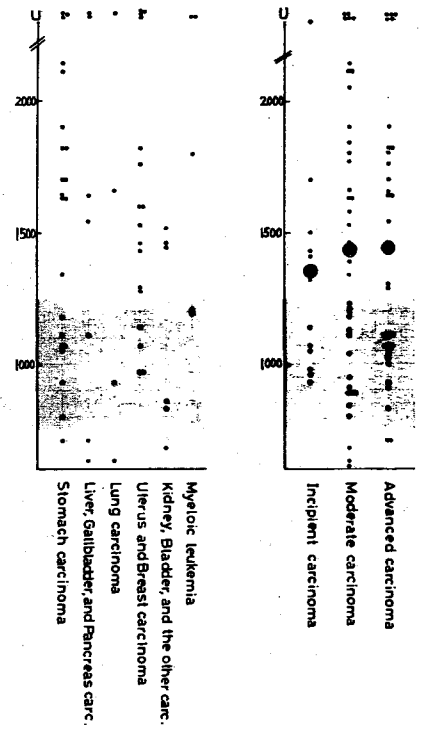

Fig. 1.

Serum β-Gase activities in various malignancies.

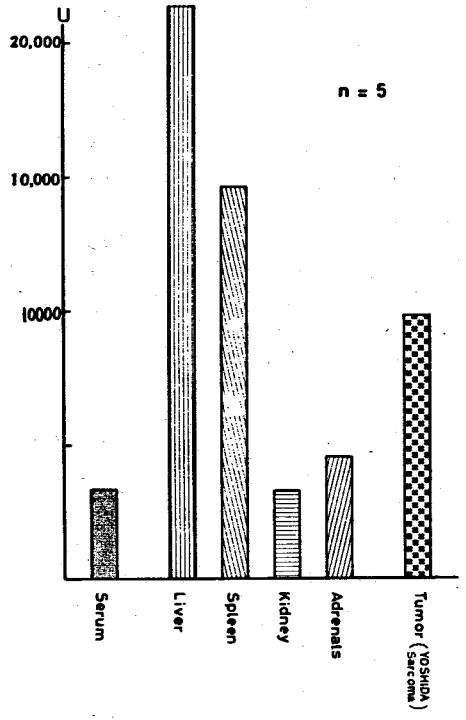

Fig. 2.

Comparison of β-Gase activities in sera, tumor tissues and in various organs.

suppression rate being 74.4% of the preadministration value. The suppression rates after GA and GAL administrations were 83.1% and 86.8%, respectively.

From the results obtained by in vitro additions of these substances into the βGase enzyme preparation, the suppression by G1:4L was attributed to the direct effect of this substance, while neither of GA and GAL showed a direct inhibiting effect upon βGase activity. When GA

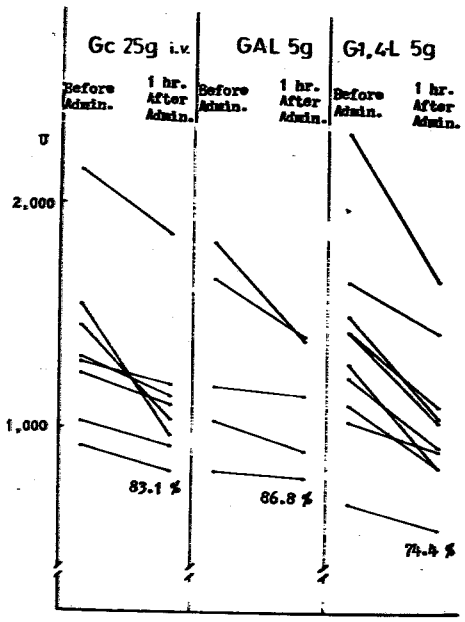

Fig. 3

Inhibiting effects of GA, GAL and of G1:4L upon β-Case activities in sera.

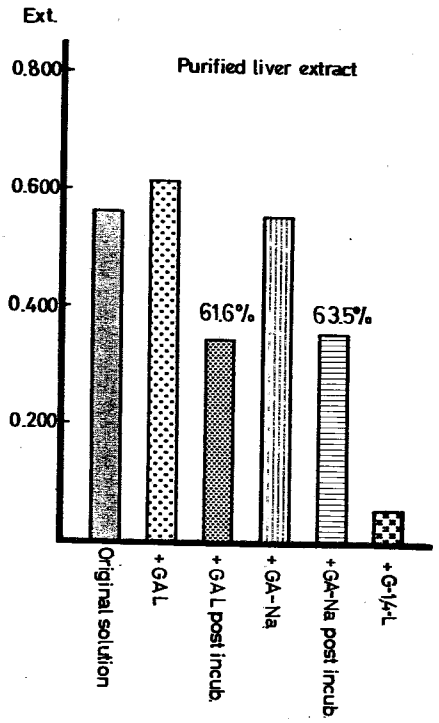

Fig. 4.

Inhibiting effect of GA, GAL and of G1:4L upon β-Case activity in vitro.

or GAL was added to the liver homogenate in which GAL-dehydrogenase was presumably involved according to Marsh's report (7), and when the mixture was incubated overnight at the optimal pH condition for GAL-dehydrogenase, i.e., pH 6.5 (8), prior to the assay of βGase inhibiting effect, the incubation mixture turned out to exert an inhibiting effect upon βGase activity (Fig. 4). The post-incubation values were 61.6% in the case of GAL, and 63.5% in the case of GA, when compared to the preincubation

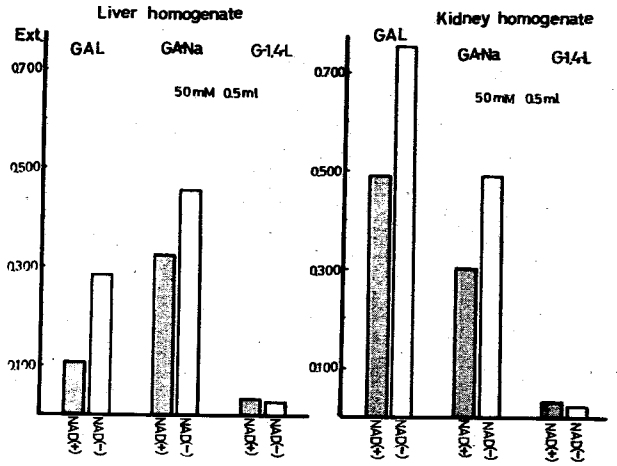

Fig. 5. Production of acid-potentiated β-Gase inhibitor when GA, GAL and G1:4L were added to tissue homogenates.

values in each case. A more remarkable inhibition was observed when NAD solution was added to the mixture (Fig. 5).

Urinary excretions of the βGase inhibitor after GA, GAL and G1:4L administrations were also assayed using the method of heat-in-acid treatment of urine. The most remarkable excretion of the inhibitor was seen in the case of GAL administration, while the inhibiting effect of urine after G1:4L administration was unexpectedly low (Fig. 6).

It was confirmed, on the other hand, that G1:4L was excreted not only into urine, but also into bile (Fig. 7).

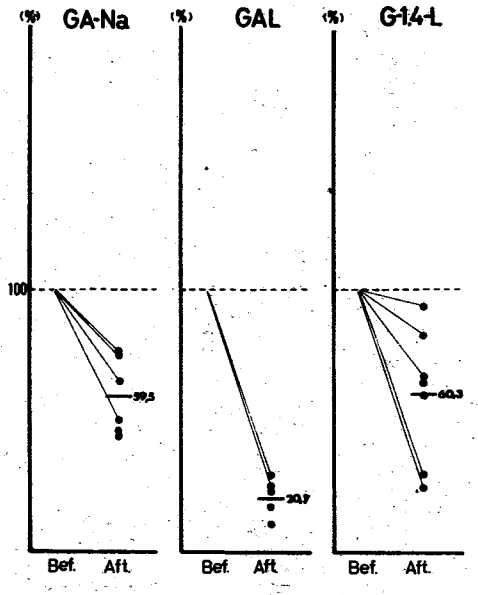

Fig. 6.

Excertion of acid-potentiated β-Gase inhibitor in 24 hr. urine after GA, GAL and G1:4L administration.

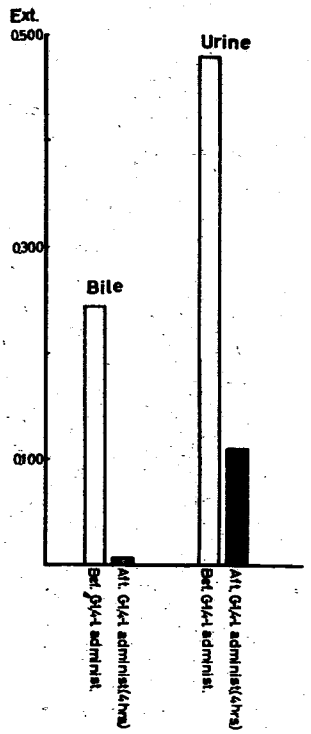

Fig. 7.

Excretion of acid-potentiated β-Gase inhibitor in bile and urine after G1:4L administration in dog.

3. Influences of GA and of G1:4L upon Tumor-Bearing Animals

Serum βGase activities of the four groups of rats were compared between each other. The most remarkable suppression of βGase was observed in animals of group D which were treated with G1:4L (Fig. 8). The suppressions of βGase activities were also found in both group B

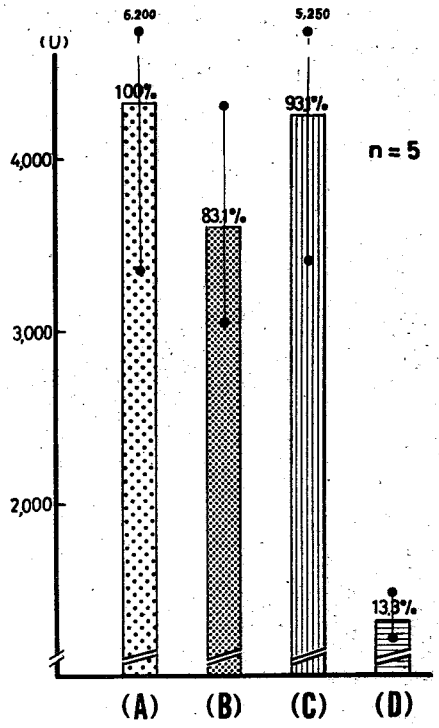

Fig. 8.

Influences of GA and G1:4L administration upon β-Gase activities in sera of rats with subcutaneously transplanted Yoshida-Sarcoma.

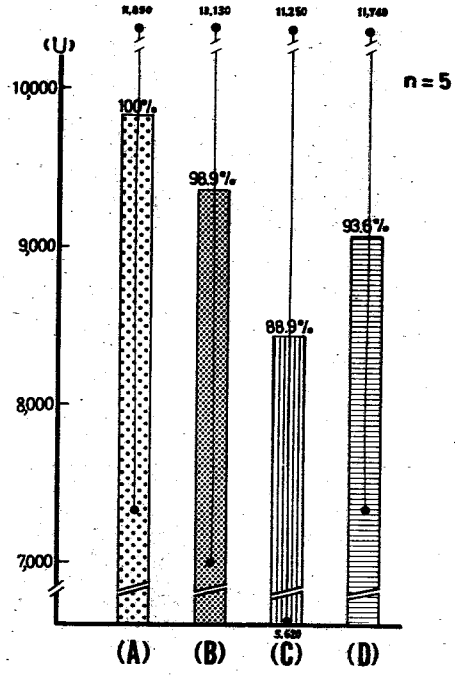

Fig. 9.

Influences of GA and G1:4L administration upon β-Gase activities in tumor tissue homogenates of rats with subcutaneously transplanted Yoshida-Sarcoma.

and group C when compared with the activities of the animals of group A, although the suppression rates were less than that of the animals of group D.

As to the βGase activities of the tumor tissues, the suppression in animals of group D was not remarkable in spite of the powerful inhibiting effect observed in serum samples of the same group of animals (Fig. 9). There were considerable decreases of the βGase

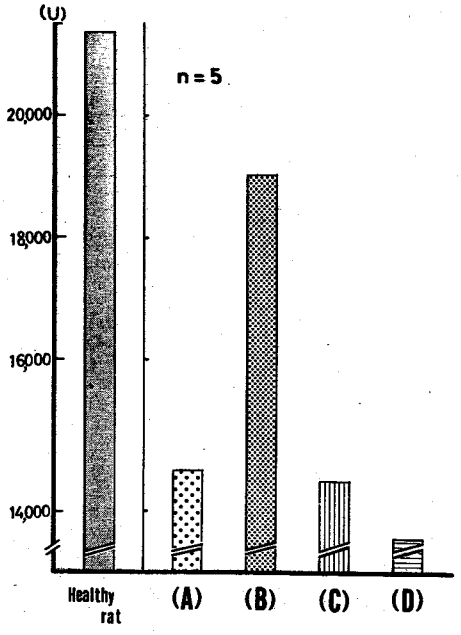

Fig. 10. Influences of GA and G1:4L administration upon β-Gase activities in the liver homogenates of rats with subcutaneously transplanted Yoshida-Sarcoma.

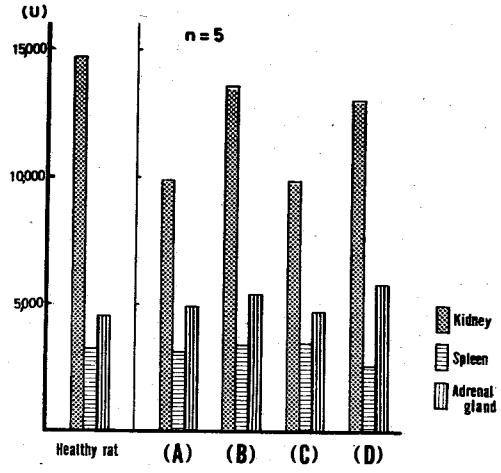

Fig. 11. Influences of GA and G1:4L administration upon β-Gase activities in organ homogenates of rats with subcutaneously transplanted Yoshida-Sarcoma.

activities of the liver in groups of rats having transplanted Yoshida sarcoma, when compared with the activities in non-transplanted healthy rats, with the exception of group B, in which the βGase activities of the liver showed subnormal values. The decrease of βGase activities of the spleen, kidney and adrenals was similar to that of the liver βGase (Figs. 10 and 11).

Results obtained by the study of influences of GA and of Gl:4L administrations upon tumor growths were shown in Fig. 12. The mean tumor weight was 5.2 g in the case of group A, 3.5 g in group B, 5.2 g in group C, and 4.8 g in group D. The ratio of tumor weight to body weight in group B was the smallest of the values of the four animal groups.

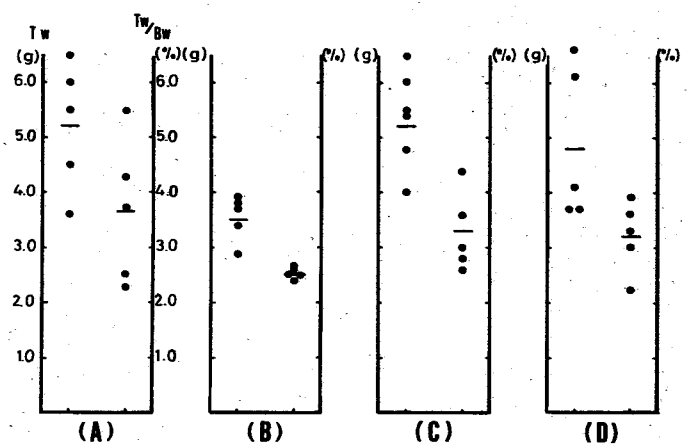

Fig. 12. Influences of GA and Gl:4L administration upon the growth of subcutaneously transplanted Yoshida-Sarcoma.

Tw: Tumor weight Bw: Body weight of rat

Discussion

Whether the demand for the detoxication by the GA system is greater or not in carcinoma hosts than in normal subjects has not been determined. From the present results showing elevations of blood GA levels and of urinary excretions of GLN in patients with carcinoma, it is assumed that the demand for detoxication through the GA metabolism is enhanced in carcinoma hosts.

On the other hand, high βGase activities in sera and in tumor tissues were observed in tumor-bearing animals, and also, elevations of serum βGase activities in patients with carcinoma were clinically observed. The enzyme βGase is known to hydrolyze O-GLN into free GA and an aglycon, while it does not affect N-GLN. The results obtained by fractional determinations of urinary GLN showed that the O-GLN excretion was not significantly high in contrast to the significantly elevated F+N-GLN excretion. These facts may suggest that the enzymic hydrolysis of O-GLN by βGase is enhanced in malignancies.

If the enhanced demand for GA and the increased O-GLN formation are indicative of the results of a teleological reaction against toxic substances produced in the body due to the malignancy, the elevation of βGase activity might be a disadvantageous reaction for the hosts.

In search of the mechanism of suppression of βGase activity, the influences of GA and of related substances upon βGase activity were observed, and it was found that serum βGase activities were suppressed in all instances (Fig. 3). Of these substances, Gl:4L showed a

direct inhibiting effect upon βGase activity (9). Although GA and GAL showed only slight inhibiting effects, incubation of the two substances with the liver homogenate and NAD resulted in potent inhibitions in both instances. The above observation may indicate a possible in vivo conversion of GA or of GAL into Gl:4L as was reported by Marsh (7). This was further confirmed by the results of determinations of the βGase inhibitor in urine after administrations of GA, GAL and of Gl:4L. In the last-mentioned experiment, the βGase inhibitor measured after the heat-in-acid treatment has been considered to be analogous to Gl:4L. The inhibiting effect of urine in the case of GAL administration was most remarkable, followed by others in the order of GA and Gl:4L, exactly contrary to the order obtained in the assay of the inhibiting effect of βGase activity of serum.

The reason why the acid-potentiated βGase inhibitor was unexpectedly low in urine, when Gl:4L was administered, will probably be explained by the fact that Gl:4L is easily metabolized into another compound in the body. The high inhibiting effect in urine when GAL was administered was probably due to the renal excretion of Gl:4L after conversion from GAL in the kidney.

These observations indicate a possibility of an in vivo conversion of GAL into Gl:4L, catalyzed by GAL-dehydrogenase present in the soluble fraction of the liver cells. Thus the demonstration of the above conversion provides evidence for an in vivo utilization of exogenous GA and GAL.

In experiments with tumor-bearing animals, the administration of GA resulted in a suppression of βGase activities in sera and in tumor tissues, and in addition, an inhibiting effect upon the tumor growth was also demonstrated. Hence the administration of GA may be considered beneficial to the host having a neoplastic disease.

Although the significance of serum βGase activity and the mechanism of its elevation in cancer remain obscure, it is assumed that the elevation of serum βGase activity is related to the increased βGase activity in tumor tissues rather than to that in the liver or other organs. Inasmuch as the decrease of GAL dehydrogenase activity in the liver of tumor-bearing animals has been reported in the literature (10), there is a possibility that the conversion of GAL to Gl:4L decreased, resulting in a lowering of the level of the βGase inhibitor which indirectly results in the elevation of βGase activity in malignancy.

The metabolic pathway of GA is represented in Fig. 13. It is known that Gl:4L is a specific potent inhibitor of βGase, while the inhibiting action of glucaric acid is far weaker than that of the former substance (8). Moreover, Gl:4L is derived from glucarate in acidic conditions around pH 4.0–4.7 (8), which is the optimal pH for the βGase activity, while glucarate can be converted from GAL by the action of GAL dehydrogenase at around pH 6.5 (8). Therefore, when O-GLN is hydrolyzed to free GA by βGase at around pH 4.5, a potent inhibition of the βGase activity can be caused by Gl:4L derived from

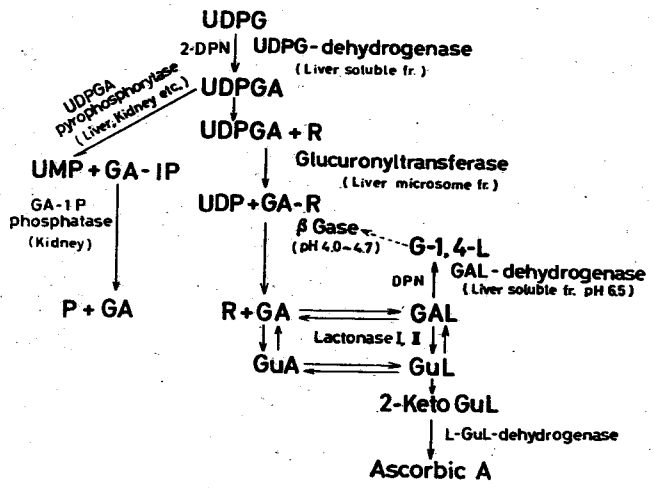

Fig. 13. Metabolic pathway of glucuronic acid (GA) and the related enzymes.

the glucarate. On the other hand, GA or GAL liberated by the enzymic hydrolysis from O-GLN may be stored as glucarate at around pH 6.5 which is the optimal condition for the GAL dehydrogenase activity, and the glucarate thus formed can be converted into G1:4L at a pH value optimal for the βGase activity. Hence the conversion of G1:4L to the glucarate and vice versa in different milieus may exert a regulating mechanism of the UDPGA-βGase system.

G1:4L has been administered as an anti-tumor agent to patients with carcinoma of the bladder by Boyland (11), and also to animals with experimental tumors by Carr (12). According to the results obtained by the present study, the administration of GAL may be more rational than

the administration of Gl:4L for the management of carcinoma of the bladder, inasmuch as higher urinary excretion of the βGase inhibitor is obtained by the former administration than by the latter. Both the amount of UDPGA and the elevation of glucuronyl transferase activity are directly related to the promotion of GLN formation (13-15), while the amount of Gl:4L and the elevation of GAL dehydrogenase activity, both of which participate in the inhibition of βGase activity, are indirectly related (7,8). Much of the relation between the GA metabolism and βGase activity in malignancy remains an enigma, and there are still many unsolved problems concerning the mechanism of the elevation of serum βGase activity as well as the significance of the elevation. Analyses of the factors related to the GA metabolism, such as UDPGA, glucuronyl transferase, βGase, GAL dehydrogenase activities in serum, and in the liver, kidney and tumor tissues are in progress using tumor-bearing animals.

Summary

Studies were carried out on the characteristic change in glucuronic acid (GA) metabolism in cancer hosts, and the interrelation between the said change and the change of serum and tissue β-glucuronidase (βGase) activities in tumor-bearing conditions was investigated both clinically and experimentally.

In patients with carcinoma, serum GA levels as well as glucuronide excretions were higher than in normal subjects, and an enhanced demand for detoxication with the GA system was suggested in carcinoma

hosts. It was also found that, in tumor-bearing animals, βGase activities both in sera and in tumor tissues were elevated, and that the enzymic hydrolysis of O-glucuronide by βGase was also enhanced in malignancies.

Serum βGase activities were suppressed in all instances after administration of GA (intravenous), glucuronolactone (GAL, oral), and of glucaro-1, 4-lactone (Gl:4L, oral). The most prominent urinary excretion of βGase inhibitor was observed after administrations of GAL.

Gl:4L exerted a direct *in vitro* inhibiting effect upon βGase activity, while GA and GAL participated in the inhibition of βGase probably through an *in vivo* conversion into Gl:4L, in accordance with Marsh's report.

Administration of GA resulted in suppressions of βGase activities not only in serum but also in tumor tissues, and it was noted that GA administrations resulted in an inhibition of the experimental tumor growth.

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