A role of asialoglycoproteins for plasma-membrane-induced inhibition of the switching from α_1 to β subtypes in adrenergic response during primary culture of rat hepatocytes

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Adrenergic responses of rat hepatocytes were studied by measuring Ins(1,4,5) P_3 (for the response via α_1 -subtype receptors) and cAMP (for β -subtype response) generation during brief incubation of cells with respective agonists. Hepatocytes from young rats with an age of 1 week displayed a very high β response without a significant α_1 response. The β response decreased and the α_1 response increased progressively as the age increased; the response was almost exclusively via α_1 receptors in hepatocytes of adult rats 9 weeks or more old. The β response developed, again at the expense of the α_1 response, in hepatocytes from adult rats during the primary culture at low cell densities [(1–2.5) $\times 10^4$ cells/cm²]. Such ' α_1 to β subtype switching' of adrenergic responses *in vitro* was totally inhibited by adding plasma mem-

branes prepared from adult rat liver into the low-cell-density culture, but not inhibited at all by membranes from young rat liver. The inhibitory effect of adult rat liver membranes was lost when the membranes had been exposed to endoglycosidase F or β -galactosidase but was not affected by prior treatment with sialidase. On the contrary, young rat liver membranes became inhibitory to ' α_1 to β subtype switching' after prior treatment with sialidase. Thus glycoproteins with unsialylated galactosyl termini on the surface of adult rat hepatocytes are likely to function as a determinant of the relative development of α_1/β subtypes of adrenergic responses; the β response is predominant in hepatocytes in the juvenile, presumably as a result of sialylation of the galactosyl termini of the functional glycoproteins.

INTRODUCTION

Adult rat hepatocytes respond to adrenergic agonists via α_1 -adrenergic receptors almost exclusively despite the occurrence of β_2 -adrenergic receptors as well on the cell surface. Rat hepatocytes can display β -adrenergic responses, however, under particular conditions including in fetal or newborn [1–5] animals, regeneration from surgical [6–10] or chemical [11] injuries, and extirpation of certain endocrine glands [12,13]. The shift can occur between the α_1 and β subtypes of adrenergic response in rat hepatocytes *in vivo* under these conditions. A similar shift would be possible even *in vitro*; β -adrenergic responses developed progressively during primary culture of adult rat hepatocytes in which α_1 responses had been predominant before culture [14–21]. α_1 -Adrenergic responses exhibited reciprocal diminution during the culture.

We have recently reported that adrenergic responses of the β -subtype developed, at the expense of α_1 -subtype responses, during culture of rat hepatocytes only if the cells were cultured at a low cell density of (1–2.5) $\times 10^4$ cells/cm² [22]. Such ' α_1 to β subtype switching' (henceforth referred to thus for brevity) was prevented efficiently when either the cell density was increased beyond this range or plasma membranes partly purified from adult rat liver were added at the start of the low-cell-density culture. It is thus strongly suggested that cell-to-cell contact through plasma membranes functions as an important factor for determination of α_1 or β subtypes in hepatocyte adrenergic responses. The purpose of the present communication is to show a role of glycoproteins on the cell surface in the inhibition of ' α_1 to β subtype switching' during primary culture of rat hepatocytes.

EXPERIMENTAL

Hepatocyte culture and the subsequent assays for α_1 - and β -adrenergic responses

Livers from rats of various ages from 1 to 17 weeks were perfused with collagenase to prepare hepatocytes, which were immediately cultured at the low cell density of (1–2.5) $\times 10^4$ cells/cm² as described previously [22]. Minor modifications of the culture conditions are as follows. Cells were cultured first for 1 h in William's E medium (ICN Flow) containing 5% (v/v) fetal calf serum (Gibco) to cause their firm attachment to dishes, and then further cultured in the same medium not supplemented with serum. Where indicated, the serum-free medium was fortified with liver plasma membranes that had been prepared and submitted to various enzyme treatments as described below. Other additions shown in Figures 5 and 6 were also made to the serum-free medium.

The β -adrenergic response of the hepatocyte monolayers thus cultured was assayed on the basis of increases in the cellular content of cAMP during the subsequent 10 min incubation of the monolayers with 100 μ M isoprenaline in the presence of 0.2 mM 3-isobutyl-1-methylxanthine. Isoprenaline was replaced by 1 μ M glucagon (Sigma) in some experiments in Figure 5. The α_1 response was based on the incorporation of ³H into cellular Ins(1,4,5) P_3 during a 20 s incubation of the *myo*-[2-³H]inositol-loaded cell monolayers with 100 μ M phenylephrine and 15 mM LiCl. Detailed procedures were described previously [22].

Plasma membrane preparations and their enzyme treatments

Livers excised from male rats of various ages were submitted to the procedure previously described [22] to obtain plasma mem-

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brane preparations. In experiments shown in Figure 2, the H4IIE hepatoma cells (from ATCC), grown in large dishes (150 mm in diameter) containing Dulbecco's modified Eagle's medium supplemented with 7.5% (v/v) fetal calf serum and the same antibiotics as for hepatocyte culture, were the source of membranes prepared by the same procedure.

Where indicated, the liver plasma membranes (100 µg of protein) were exposed to 1 unit/ml β -galactosidase [from *Escherichia coli* (EC 3.2.1.23); Boehringer Mannheim] for 12 h, 2 units/ml endoglycosidase F [from *Flavobacterium meningosepticum* (EC 3.2.1.96); Boehringer Mannheim] for 12 h, 1 unit/ml sialidase [from *Arthrobacter ureafaciens* (EC 3.2.1.18); Nakaraiteque, Tokyo, Japan] for 16 h or 1% (w/v) trypsin for 10 min at 37 °C in 2 ml of the incubation medium. The compositions of the media used were: 0.1 M sodium acetate buffer, pH 5.7, containing 0.5 mM CaCl_2 for β -galactosidase; 20 mM Hepes, pH 7.6, containing 150 mM NaCl, 2 mM EDTA and 1 mM PMSF for endoglycosidase F; 0.2 M sodium phosphate buffer, pH 6.0, containing 0.5 mM CaCl_2 for sialidase; and Krebs-Ringer Hepes buffer, pH 7.4, for trypsin. After these treatments the reaction mixture was centrifuged at 12000 *g* for 5 min at 4 °C and the resultant pellet was washed three times with ice-cold Hepes-buffered saline, pH 7.4, to be immediately added to the hepatocyte culture. Exposure of the plasma membrane to heat (in a boiling water bath for 3 min) was achieved in 0.2 M PBS, pH 7.4, followed by centrifugation at 1500 *g* for 5 min at 4 °C and a repeated washing of the pellet.

Measurement of [^3H]thymidine incorporation

Mitogenesis was measured by adding 1 µM [^3H]thymidine (1 µCi/ml, NEM) into hepatocytes that had been cultured first for 24 h under the standard conditions with or without plasma membranes and then for a further 20 h with 100 nM insulin (Sigma) and 20 ng/ml epidermal growth factor (Toyobo, Tokyo, Japan) in the fresh medium. The radiolabelling was terminated at 10 h by the addition of 0.2 mg NaN_3 (0.2 ml) followed by chilling in ice/water for 10 min and by further addition of a 4:1 mixture of methanol and acetic acid. The fixed cell layer, separated from the supernatant after 10 min, was kept in 2 ml of the same mixture for an additional 10 min before being washed three times with cold 2% (v/v) perchloric acid and once with water. The radioactivity of the acid-insoluble material was quantified by liquid-scintillation spectrophotometry.

Sources of reagents

Reagents for radioimmunoassay of cAMP were gifts from Yamasa Shoyu Co. Trypsin, fetuin, asialofetuin and lactosylceramide were purchased from Sigma. Sources of other reagents, except for those described above, were the same as described previously [22].

RESULTS

Age dependence of ' α_1 to β subtype switching' of hepatocyte adrenergic response and roles of plasma-membrane proteins

Hepatocytes were prepared from livers of rats of various ages from 1 to 17 weeks and submitted to primary culture at a low cell density of 10^4 cells/cm 2 . The cell monolayers thus cultured, together with the cell suspensions before culture (culture time 0), were then incubated for a short time with isoprenaline or phenylephrine to measure cAMP production as a β -adrenergic response (Figure 1a) or InsP_3 formation as an α_1 -adrenergic response (Figure 1b) respectively. Before culture, the hepatocytes

Figure 1 Age-dependent predominance of α_1 - or β -adrenergic response of rat hepatocytes and progressive subtype switching of the response during subsequent primary culture

Hepatocytes from rats aged 1 (○), 2 (●), 3 (△), 5 (▲), 9 (□) or 17 (■) weeks were cultured at a low cell density of 10^4 cells/cm 2 for periods of time indicated on the abscissa. These cells were then submitted to an assay of β response (a) or α_1 response (b) on the basis of isoprenaline-induced cAMP or phenylephrine-induced $\text{Ins}(1,4,5)\text{P}_3$ generation respectively, as described in the Experimental section. Representative data are shown from experiments repeated two or three times with similar results.

from the youngest rat liver (1 week old) displayed an extremely high β response and a very low or essentially no α_1 response. The β response decreased and the α_1 response increased progressively as the age of the cell-donor rats increased, until very small β response with very high α_1 response was obtained at the adult age of 17 weeks. Thus the predominance of α_1 over β responses is characteristic of adult rat hepatocytes, whereas exactly the reverse situation is unique to adrenergic responses of juvenile rat livers.

The very high β response and the very low α_1 response of the hepatocytes of rats with ages below 3 weeks underwent no further significant changes during 24 h hepatocyte culture at a low cell density. When the β and α_1 responses were further lowered and enhanced respectively as the age of rats increased beyond 5 weeks, there were progressive recoveries of the responses during the 24 h culture. As a result, the β and α_1 responses of hepatocytes of these growing or adult rats attained their respective maximal and minimal values after the 24 h culture; the former became predominant over the latter in the cultured adult rat hepatocytes (Figure 1). These culture-induced changes in the adrenergic response were unique to the low-cell-density culture; there was no such a change during culture when the cell density was increased to 10^5 cells/cm 2 (results not shown), in agreement with our previous report [22].

The reciprocal relationship between α_1 and β responses, i.e. the fact that a high α_1 response was invariably accompanied by a low β response and vice versa, holds true for all the hepatocytes excised from rats of various ages or submitted to the low-cell-density primary culture. It should be noted that the effects *in vitro* of the culture to increase the β response and to decrease the α_1 response were never 'additive' to the same effects *in vivo* of decreasing the age of the liver-donor rats. In other words, the respective maximum and minimum values (i.e. points towards the right of plots in Figure 1) for β and α_1 responses obtained *in vitro* after a 24 h culture of hepatocytes from the rats older than 5 weeks never exceeded the corresponding values (points on the left ends of plots) *in vivo* of the younger (1–3 weeks old) rats. It is therefore very likely that virtually the same mechanism is

Figure 2 Effects of plasma membrane addition on development of β -adrenergic response during hepatocyte culture

Adult (10–12 weeks old) rat hepatocytes were cultured at a low cell density of 10^4 cells/cm² for 24 h with the addition of increasing concentrations of plasma membranes as shown on the abscissa. The cultured cells were then submitted to β -adrenergic response measurement as in Figure 1a. The sources and treatments of plasma membranes are: (a) adult rat liver, without further treatment (○), or with boiling for 3 min (●) or trypsin treatment (△); (b) from adult rat liver (○), from hepatoma H4IIE (●), from livers of rats aged 1 week (△) or 3 weeks (▲) without further treatment in all cases. Typical data are taken from experiments repeated three or more times with essentially the same results.

involved in determining relative development of α_1 and β responses *in vivo* (rat growth) and *in vitro* (hepatocyte culture).

The increase in β response (and the reciprocal decrease in α_1 response) occurring during low-cell-density culture of adult-rat hepatocytes was inhibited by increasing the cell density [22]. The inhibition was mimicked by the addition to the low-cell-density culture of plasma-membrane preparations from adult rat liver, thereby leading us to consider that cell adhesion through plasma membranes is responsible for the inhibition observed in the high-cell-density culture (see [22]). The involvement of membrane proteins in the inhibition of β response development during culture is suggested by the results in Figure 2a, where the progressive inhibition elicited by increasing amounts of membranes from adult rat liver was totally reversed by previously submitting the membranes to typical procedures of protein denaturation or decomposition such as heating and tryptic digestion.

The inhibitory effect on β response development was a characteristic unique to membranes prepared from adult rat liver. No inhibition was caused by membrane preparations from hepatoma (Figure 2b). Likewise, liver membranes from young rats only 1 or 2 weeks of age, on addition into the low-cell-density culture, did not provoke significant inhibition of the culture-induced development of β responses. The magnitude of the small inhibition by these young rat liver membranes, although seemingly not significant, was larger for 2-week-old rats than for 1-week-old rats (Figure 2b), suggesting an inverse correlation with magnitudes of the hepatocyte β responses of these young rats (Figure 2a). Membrane proteins could play important roles in age-dependent relative occurrences of α_1 - and β -adrenergic responses of hepatocytes.

Role of glycoproteins as a determinant of ' α_1 to β subtype switching' during hepatocyte culture

Plasma membranes prepared from adult rat livers were exposed to enzymes catalysing modifications of oligosaccharide side

Figure 3 Modification by prior glycosidase digestion of ability of adult rat liver membranes to inhibit ' α_1 to β subtype switching' during hepatocyte culture

Adult rat hepatocytes were cultured at a low cell density of 10^4 cells/cm² for periods of time shown on the abscissa without (○) or with the addition of plasma membranes (0.2 mg/ml protein). The membranes prepared from adult rat livers had been exposed to buffer solely (●), β -galactosidase (△), sialidase (▲) or endoglycosidase F (■) immediately before the addition. The hepatocytes thus cultured were submitted to β -response (a) or α_1 -response (b) assay as in Figure 1. Similar results were obtained in other three or more experiments with the same design.

chains of glycoproteins. Rapid progressive increases of the β response and rather slow progressive decreases of the α_1 response during low-cell-density culture of adult rat hepatocytes were both abolished when the culture medium was supplemented with membranes from adult rat livers. Prior treatment of these membranes with sialidase had no effect on the membrane-induced abolition of the ' α_1 to β subtype switching' (Figure 3). In sharp contrast, the membrane preparations that had been exposed to β -galactosidase or endoglycosidase F were no longer capable of abolishing the increases in β response and the decreases in α_1 response during the culture. There were almost normal changes, although slightly lower than in the culture without membranes, of α_1 and β responses during the culture in the presence of membranes treated with β -galactosidase or endoglycosidase F. Thus membrane asialoglycoproteins terminating with β -galactosides are likely to play an essential role in inhibition of culture-induced changes of α_1 - and β -adrenergic responses.

The failure of membranes prepared from juvenile rat livers to inhibit the development of the β response during the low-cell-density hepatocyte culture as first observed in Figure 2b is reproduced in Figure 4a. In addition, the culture-induced progressive decrease in α_1 response was observed normally in either the presence or the absence of the membranes from young rat livers in the culture medium (Figure 4b). No significant changes were observed in plots of the positive/negative developments of β/α_1 responses in Figures 4a and 4b respectively when the membranes from young rat liver had been incubated with β -galactosidase or endoglycosidase F. Unexpected results were the effects of prior exposure to sialidase of the membranes from young rat liver: sialidase pretreatment provided the young rat liver membranes with a novel ability to resemble that of untreated adult rat liver membranes or to suppress almost totally the culture-induced changes in α_1 and β responses. The sialidase treatment of young rat liver membranes might have unmasked the β -galactoside termini of cell surface glycoproteins that are essential in the prevention of ' α_1 to β subtype switching' during the low-cell-density culture of adult rat hepatocytes. In agreement

Figure 4 Inhibition of ‘ α_1 to β subtype switching’ during hepatocyte culture by addition of young rat liver membranes previously exposed to sialidase

Adult rat hepatocytes were cultured at a low cell density of 10^4 cells/cm² for periods of time shown on abscissa without (○) or with the addition of plasma membranes (0.2 mg/ml protein) prepared from young (1 week old) rat livers. The membranes had been exposed to buffer solely (●), β -galactosidase (△), sialidase (▲) or endoglycosidase F (■) immediately before the addition. The hepatocytes thus cultured were submitted to β -response (a) or α_1 -response (b) assay as in Figure 1. The data are representative from experiments performed more than three times with similar results.

with this view, significant changes in α_1/β responses took place during the culture with the young rat liver membranes that had been exposed consecutively to sialidase and β -galactosidase (results not shown).

Role of the β -galactoside termini of membrane glycoproteins in inhibition of DNA synthesis during hepatocyte culture

The rate of DNA synthesis, as measured by [³H]thymidine incorporation in the presence of growth factors, during hepatocyte culture was much higher in the cells cultured at low cell densities than in those cultured at high cell densities. The lower rate of DNA synthesis during the high-cell-density culture was mimicked by adding adult rat liver membranes into the low-cell-density culture (Y. Kajiyama & M. Ui, unpublished work). Table 1 shows DNA synthesis in the S phase of the cell cycle, i.e. during the final 10 h of a 30 h low-cell-density culture with insulin and

Figure 5 Antagonism between glyco-compounds and adult rat liver membranes as regards β response development during hepatocyte culture

Adult rat hepatocytes were cultured at a low cell density of 2.5×10^4 cells/cm² for 8 h with increasing concentrations of asialofetuin (a), lactose (b) or lactosylceramide (c) as shown on the abscissa. The culture medium was further supplemented (●, ■) or not (○, □) with plasma membranes (0.2 mg/ml protein) from adult rat livers. These cultured cells were then submitted to assays of cAMP-generating response to isoprenaline (○, ●) or glucagon (□, ■) as described in the Experimental section. Representative data are shown from experiments repeated twice with similar results.

epidermal growth factor. Just as in ‘ α_1 to β subtype switching’ shown in Figures 2–4, DNA synthesis in the S phase of cells cultured at low density was efficiently suppressed by the addition of adult rat liver membranes (see the first row in the third column of figures). The young rat liver membranes were also without effect in this regard (the first row in the first column of figures).

The essential role of the β -galactoside termini of membrane glycoproteins was suggested by the loss of the above-mentioned function of adult rat liver membranes on prior treatment of the membranes with β -galactosidase or endoglycosidase F. Pre-treatment with sialidase was without effect on the adult rat liver membranes, as had been expected from Figure 3 (Table 1). In young rat liver membranes, sialidase treatment was the sole means of providing the membranes with an ability to inhibit DNA synthesis. The treatment with β -galactosidase or endo-

Table 1 Mitogenesis of cultured rat hepatocytes as influenced by addition of the plasma membranes prepared from adult rat or young rat livers and treated with glycosidases

Adult rat hepatocytes were cultured at a low cell density of 2.5×10^4 cells/cm² to follow the incorporation of [³H]thymidine, as a measure of DNA synthesis, as described in the Experimental section. The hepatocyte culture was achieved with the addition of plasma membranes (0.2 mg/ml protein) from young (1 week old) or adult (8 weeks old) rat livers. DNA synthesis in hepatocyte culture without plasma membranes was $(57.3 \pm 3.8) \times 10^{-3}$ d.p.m. per mg of protein. The plasma membranes were added to the culture medium without prior treatment (‘No treatment’) or after incubation with glycosidases as recorded on the left of the table. The incubation in the medium containing no enzyme is shown by ‘Mock’. Each result represents the mean $\pm$ S.E.M. ($n = 6$ for ‘without membranes’; $n = 3$ for others) within a typical experiment, which was repeated more than three times with similar results.

Membrane treatment	$10^{-3} \times$ DNA synthesis (d.p.m. per mg of protein)			
	With membranes from young rats		With membranes from adult rats	
	Mock	Treated	Mock	Treated
No treatment	56.2 $\pm$ 0.8		10.0 $\pm$ 4.9	
Sialidase	53.6 $\pm$ 1.2	10.8 $\pm$ 8.2	14.1 $\pm$ 4.1	11.8 $\pm$ 7.0
Endoglycosidase	52.4 $\pm$ 2.8	61.7 $\pm$ 0.3	16.5 $\pm$ 4.8	59.2 $\pm$ 5.8
β -Galactosidase	56.5 $\pm$ 3.0	59.9 $\pm$ 2.6	11.2 $\pm$ 3.5	60.1 $\pm$ 3.8

Figure 6 Antagonism between glyco-compounds and adult rat liver membranes as regards mitogenesis during hepatocyte culture

Adult rat hepatocytes were cultured at a low cell density of 2.5×10^4 cells/cm², to follow [³H]thymidine incorporation as described in the Experimental section, with increasing concentrations of asialofetuin (a), lactose (b) or lactosylceramide (c) as shown on the abscissa. The culture medium was further supplemented (●, ▲) or not (○, △) with plasma membranes (0.2 mg/ml protein) from adult rat livers. In (a) the effect of fetuin, added instead of asialofetuin, is shown (▲, △). Each point represents the mean $\pm$ S.E.M. for triplicates within a typical experiment that was repeated twice with similar results.

glycosidase F exerted no effect on the young rat liver membranes (Table 1). Thus a similar mechanism might underlie, or the same molecules might be involved in, the inhibitions of ' α_1 to β subtype switching' and S-phase DNA synthesis.

Antagonistic effects of glycoconjugates having β -galactosyl termini on the functions of adult rat liver membranes

An oligosaccharide and two glycoconjugates each with a β -galactosyl terminus, i.e. lactose, lactosylceramide and asialofetuin, were studied for their ability to antagonize the effects of membranes from adult rat liver during a low-cell-density culture of adult rat hepatocytes (Figures 5 and 6). As reported previously [22], cAMP-generating responses of hepatocytes to glucagon exhibited no detectable change during culture either with or without membrane preparations. The three glyco-compounds were also without effect on the response to glucagon. The development of the β response during 8 h of culture was completely inhibited by the addition of adult rat liver membranes. The inhibition was progressively reversed as concentrations of the glyco-compounds in the culture medium were increased, until essentially no inhibition was observed at approx. 50 μ g/ml (Figure 5). The order of potency is thus asialofetuin > lactosylceramide > lactose on the basis of the molecular mass of the glyco-compounds. Conceivably, an attached protein or large conjugate could afford favourable conditions for binding of a β -galactosyl moiety to the target on the cell surface.

Inhibition of the S-phase DNA synthesis provoked by adult rat liver membranes was also progressively attenuated as concentrations of the glyco-compounds were increased in the culture medium (Figure 6). Inhibition was overcome when the culture medium was supplemented with asialofetuin at 60 μ g/ml or more. Fetuin with β -galactoside termini sialylated, unlike asialofetuin, did not affect membrane-induced inhibition even at the highest concentration of 100 μ g/ml, equivalent to twice the minimally effective concentration of asialofetuin (Figure 6a). An

essential role of unmasked β -galactosyl termini of glycoproteins is thus strongly suggested.

DISCUSSION

The relative occurrence of adrenergic receptor-mediated signalling via the α_1 and β_2 subtypes in hepatocytes must be under physiological and pathological control, as revealed by the overwhelming predominance of one over the other under particular conditions *in vivo* and *in vitro* (see the Introduction section for previous references). One of the typical examples *in vivo* is shown in Figure 1, where the α_1 -subtype response is predominant over the β -subtype response in hepatocytes of adult rats, in complete contrast with those from juvenile rats. Figure 1 is also a good example of the systems *in vitro*: ' α_1 to β subtype switching' or development of β response at the expense of the previously overwhelming α_1 response occurred during low-cell-density culture of the hepatocytes from rats older than 5 weeks. These changes in α_1 -subtype and β -subtype responses *in vitro* during hepatocyte culture were very marked: the β response increased more than 5-fold and the α_1 -response decreased to one-third after a 24 h culture of adult (older than 9 weeks) rat hepatocytes. However, the final level of responses thus attained after the culture did not exceed the responses of hepatocytes from younger rats. Common mechanisms or common molecular factors must therefore be involved in the determination of the relative occurrence of α_1 and β responses *in vivo* and *in vitro*. Thus the hepatocyte primary culture affords a good experimental system for the study of molecular mechanisms whereby ' α_1 to β subtype switching' of adrenergic responses takes place under physiological and pathological conditions.

The inclusion of adult liver membranes in the medium of low-cell-density culture of adult rat hepatocytes provided conditions very similar to the high-cell-density culture of the same hepatocytes in that there was no ' α_1 to β subtype switching' in either case. It would therefore be reasonable to assume that the cell-to-cell contact via plasma membranes that inevitably occurs during high-cell-density culture and that is mimicked by the addition of adult liver membranes to the low-cell-density culture is responsible for the inhibition of ' α_1 to β subtype switching' under these conditions. The cell-surface glycoproteins are involved in cell-cell contact, as evidenced by the data in Figure 3 showing that membrane-induced inhibition of ' α_1 to β subtype switching' was no longer observable after the membranes had been exposed to endoglycosidase F. The effect of endoglycosidase F in this regard might imply that N-linked oligosaccharides are involved. The implication remains to be studied further, although the reversal by lactose of membrane-induced inhibition of the subtype switching (Figure 5) might suggest an important role of terminal sugars. In fact, the particular role of galactose-terminated glycoproteins was shown by the ability of β -galactosidase to abolish membrane-induced inhibition of the subtype switching (Figure 3).

In contrast, adult liver membranes pretreated with sialidase inhibited the subtype switching to the same extent as the untreated membranes (Figure 3). Increasing the amount of sialidase-treated adult liver membranes in the culture medium gave a progressive inhibition pattern of subtype switching that was not significantly different from the pattern obtained with untreated membranes (results not shown). Most of the plasma-membrane glycoproteins are sialylglycoproteins in adult rat hepatocytes [23]. Terminal galactose residues must have appeared on treatment with sialidase, as evidenced for the hepatoma cell line HepG2 [24]. Nevertheless, adult rat liver membranes inhibited ' α_1 to β subtype

switching' to the same degree either before or after loss of sialic acid. The terminal β -galactosyl moieties of particular glycoproteins, rather than all galactose-terminal glycoproteins, are likely to play their selective role in the cell-cell interaction responsible for the inhibition of ' α_1 to β subtype switching' of hepatocyte adrenergic responses.

For their function to be displayed, the galactose-terminated glycoproteins whose presence on adult liver membranes is suggested should be recognized by a binding protein or a receptor protein on the surface of cultured adult rat hepatocytes. Mammalian hepatocytes are known to possess such galactose receptors, of which the major one is the asialoglycoprotein receptor whose principal function seems to be the clearance of serum glycoproteins that have lost their terminal sialic acid residue [25,26]. In fact, asialofetuin acts as a good ligand (see Figures 5 and 6) for the asialoglycoprotein receptor [27], which is involved in adhesion of rat hepatocytes to matrices containing covalently attached galactosyl groups [28]. The asialoglycoprotein receptor is itself a glycoprotein. The possibility can therefore not be excluded that homophilic bindings are involved in the cell-cell interaction of adult rat hepatocytes. The real role of galactose receptors, together with the validity of this possibility, is the subject of further investigation.

The overwhelming predominance of the β response over the α_1 response characteristic of juvenile rat hepatocytes *in vivo* (Figure 1) seems to be due, at least in part, to the inability of the membrane glycoproteins to inhibit ' α_1 to β subtype switching' observable *in vitro* (Figure 4). The young rat liver membranes became inhibitory to the subtype switching when they had been exposed to sialidase (Figure 4). It is therefore very tempting to speculate that galactose-terminated glycoproteins, which are present in adult rat liver membranes playing their inhibitory role in subtype switching as discussed above, are in the sialylated or inactive state in juvenile rat livers. The speculation is to some extent consistent with the report that all the galactose-terminated glycoproteins were sialylated in newborn rat livers and rapidly lost sialic acid as the age increased [29].

We have recently discussed [22] that development of the β response is a growth-related function of liver parenchymal cells according to Ichihara's classification [30], whereas the predominance of the α_1 response can be included in the differentiated phenotypes. Environments or manoeuvres *in vivo* and *in vitro* favouring hepatocyte proliferation, and being unfavourable for differentiation (i.e. those for livers from fetal, newborn or juvenile rats [1–5], regenerating livers after partial hepatectomy [6–9], bile-duct ligation [10] or repeated injection of carcinogens or hepatotoxic agents [11] and the primary culture of rat hepatocytes at low cell densities [14–22]), are characterized by the development of β responses at the expense of α_1 responses. In contrast, adult rat livers, before and after high-cell-density culture, display hepatocyte-specific differentiated functions as well as adrenergic responses exclusively via α_1 receptors [17, 22]. A close relationship between ' α_1 to β subtype switching' and cell growth is suggested again by this study, because the β -galactosyl terminal glycoproteins with similar susceptibilities to glycosidases seem to participate in inhibitions of both subtype switching (Figure 3) and cell proliferation (Table 1). Cell membranes from senescent or quiescent young human diploid fibroblasts, on being added to the culture medium, were reported to inhibit the passage of replicating young cells from G_1 phase to S phase [31]. The membrane-associated inhibitor was suggested to be a glycoprotein because of its sensitivity to trypsin and periodate [31].

Intriguingly, expression of the asialoglycoprotein receptor on the surface of rat hepatocytes is developmentally regulated in such a fashion that the expression is markedly reduced under

proliferative conditions, including a low-cell-density culture with growth factors [32], liver regeneration [33] and hepatocarcinogenesis [34]. This is one of the reasons why we have proposed above a possible involvement of the asialoglycoprotein receptor in the inhibition of ' α_1 to β subtype switching'. The particular glycoproteins, including asialoglycoprotein receptor itself (see above), on the cell surface could act as a ligand for the receptor (the binding sites), reflecting progressive and saturable inhibition of ' α_1 to β subtype switching' induced by increasing amounts of membrane (Figure 2). Their binding to the receptor could be antagonized by an excess amount of the normal galactose, as shown in Figures 5 and 6, being suggestive of essential roles of glyco-termini. Further quantitative analyses of β -galactosyl termini, their sialylated form and asialoglycoprotein receptors on the cell surface of adult rat and young rat livers are now in progress in our laboratory to provide evidence for their roles suggested in this study as the determinants of ' α_1 to β subtype switching' occurring *in vivo* and *in vitro*.

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