

Developmental Abnormalities in Transgenic Mice Expressing a Sialic Acid–Specific 9-O-Acetyltransferase

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Summary

9-O-acetylation of sialic acids is tissue specific and developmentally regulated. We have selectively destroyed these O-acetyl groups during murine embryogenesis by expressing the 9-O-acetyl–sialic acid–specific esterase of influenza C. DNA constructs driven by the metallothionein promoter arrested development at the 2-cell stage and gave a markedly decreased yield of live mice. A similar construct driven by the phenylethanolamine-N-methyltransferase promoter did not cause this block, but gave transgenic mice with selective expression of esterase in the retina and the adrenal gland. These organs showed variable abnormalities in organization, while all other tissues examined appeared normal. The ganglioside 9-O-acetyl-G_{D3} was selectively destroyed in target tissues. Thus, 9-O-acetylated sialic acids may play a role in murine development at the 2-cell stage and in certain differentiated tissues.

Introduction

At least several hundred enzymes are involved in the biosynthesis and modification of the diverse oligosaccharide structures found in higher animal cells (for some examples see Kornfeld et al., 1982; Kornfeld and Kornfeld, 1985; Chen et al., 1989; Schachter, 1988; Lindahl et al., 1989; Paulson et al., 1989; Higa et al., 1989). Mutants in many steps of these biosynthetic pathways are easily obtained in tissue culture cells (for some examples see Reitman et al., 1982; Huffaker and Robbins, 1983; Chaney and Stanley, 1986; Kingsley et al., 1986; Bame and Esko, 1989). However, such mutants usually do not show a remarkable phenotype *in vitro*, indicating that many oligosaccharide structures are not required for the growth and maintenance of single cell types in the environment of the tissue culture dish. On the other hand, glycosylation mutants in intact higher animals are very rare (Reitman et al., 1981; Nakazawa et al., 1984; Fukuda et al., 1987, 1990; Yamamoto et al., 1990). A likely explanation is that many specific types of glycosylation play crucial roles during embryogenesis and that mutations in the intact animal result in lethal aberrations. The developmentally regulated expression of a variety of carbohydrate structures supports this hypothesis, as does their frequent re-expression as oncofetal antigens in cancer cells (Hakomori, 1986; Feizi, 1985). Remarkable spatial and temporal changes in the expression of several oligosaccharide structures

during embryogenesis have been described (Hakomori, 1981; Feizi, 1985; Chou et al., 1989; Rutishauser, 1990; Constantine-Paton et al., 1986; Bouhours and Bouhours, 1983; Muchmore et al., 1987; Chou et al., 1990), suggesting that they could play a role in the interactions of embryonic cells and in the organization of developing tissues. To directly address this possibility it is necessary to selectively interrupt or alter glycosylation in the intact developing embryo. However, this must be done in a manner that avoids an early embryonic lethal event.

The sialic acids are a diverse family of 9-carbon sugars found as terminal monosaccharides on many vertebrate glycoconjugates (Rosenberg and Schengrund, 1976; Schauer, 1982). The hydroxyl groups of these molecules can be O-acetylated at different positions, significantly altering the size, hydrophobicity, net charge, and enzymatic susceptibility of the parent compound (Schauer, 1982; Manzi et al., 1990; Iwasaki et al., 1990; Drzeniek, 1973; Varki and Diaz, 1983; Corfield et al., 1986). O-acetyl esters at the 9 position of sialic acids show developmental regulation, tissue-specific expression, and regional distribution in a variety of systems and sometimes reappear as oncofetal antigens (Levine et al., 1986; Constantine-Paton et al., 1986; Muchmore et al., 1987; Sparrow and Barnstable, 1988; Schlosshauer et al., 1988; Mendez-Otero et al., 1988; Chou et al., 1990; Reid et al., 1984; Cheresh et al., 1984a, 1984b). For example, the 9-O-acetylated form of the disialoganglioside G_{D3} (Levine et al., 1984; Cheresh et al., 1984a, 1984b; Thurin et al., 1985) is selectively expressed in the embryonic adrenal gland, the mesonephros, and certain regions of the developing nervous system, but not in other parts of the fetus. Studies with the monoclonal antibody JONES have demonstrated that this unusual ganglioside shows a dorsal–ventral gradient across the developing retina, a distribution distinct from its precursor, G_{D3} (Constantine-Paton et al., 1986; Blum and Barnstable, 1987; Sparrow and Barnstable, 1988). Similar discordance between G_{D3} and its O-acetylated counterpart is found in other areas of the developing central and peripheral nervous system (Blum and Barnstable, 1987; Sparrow and Barnstable, 1988; Schlosshauer et al., 1988; Mendez-Otero et al., 1988; Stallcup et al., 1989). Expression of 9-O-acetyl-G_{D3} decreases shortly after birth and in the adult animal appears confined to the adrenal medulla, the renal glomerular podocyte, and a few cells in the nervous system (Blum and Barnstable, 1987; Sparrow and Barnstable, 1988; Schlosshauer et al., 1988; Mendez-Otero et al., 1988; Stallcup et al., 1989; Dekan et al., 1990; J. Reiviren, H. Holthofer, and A. Miettinen, unpublished data). In spite of such intriguing clues, little is known about the specific biological roles of these ester groups during development.

The influenza C virus has a single coat protein called the hemagglutinin-esterase, which is synthesized as a type I membrane-bound polypeptide. The mature protein has a single transmembrane hydrophobic segment near the carboxy-terminus, with only 3 amino acids protruding into

the cytosolic side of the membrane (Herrler et al., 1985; Nakada et al., 1984; Pfeifer and Compans, 1984). The hemagglutinin activity in the ectodomain binds specifically to 9-O-acetylated sialic acids (Rogers et al., 1986; Vlasak et al., 1987). However, the same protein also contains a "receptor destroying enzyme" that functions optimally at a neutral pH and specifically cleaves 9-O-acetyl esters from sialic acids (Herrler et al., 1985; Vlasak et al., 1987, 1989; Nishimura et al., 1988). The latter activity is mediated by a novel serine active site mechanism (Hayes and Varki, 1989), which can be selectively inactivated without affecting the hemagglutinin activity (Muchmore and Varki, 1987). At or above ambient temperatures the esterase is dominant over the hemagglutinin. Thus, it is impossible to demonstrate hemagglutinin activity with this virus, unless the experiment is carried out at 4°C to minimize esterase activity. In certain cell types, a tryptic cleavage of the protein also occurs, exposing a potential fusion polypeptide sequence. This fusion activity is weak compared with that of other influenza hemagglutinins (Eisenlohr et al., 1988; van Meer et al., 1985; Stegmann et al., 1986, 1987; Wharton et al., 1986, 1988) and is only functional at pH values below 5.5 (Formanowski et al., 1990). Thus, under physiological conditions (37°C and neutral pH) this protein functions only as a highly specific sialate: 9-O-acetylerase.

Among other paramyxoviruses, the activity of such "receptor destroying enzymes" is well known to eliminate virus receptors on cell surfaces on which they are expressed (Morrison and McGinnes, 1989; Els et al., 1989). We reasoned that if the highly specific influenza C esterase were to be expressed as a plasma membrane protein on cells of an intact developing embryo, it should destroy 9-O-acetyl esters of sialic acids on the same cell and perhaps on adjacent cells. Any resulting phenotype should therefore provide clues to the normal functions of these esters on the cell surface in question. Furthermore, since the activity required is enzymatic, high level expression should not be needed for significant cleavage of the O-acetyl esters to occur. We were also encouraged by a study in which direct injection of an endoneuraminidase into the developing chick retina was shown to induce developmental anomalies (Rutishauser et al., 1985). We therefore set out to develop transgenic mice (Hanahan, 1989; Palmiter et al., 1983) expressing the influenza C esterase in a tissue-specific and developmentally regulated fashion.

Results

Preparation of Constructs and Injection into Fertilized Eggs

A cDNA encoding the influenza C esterase was ligated at the 3' end to genomic sequences from the second and third exons of the human β -globin gene, which contain one intron, and the signals for polyadenylation. The β -globin exons were added because intronless cDNAs are more prone to give poor expression in transgenic animals (Brinster et al., 1988). Previously described promoter regions from either the mouse metallothionein gene (Brinster et al., 1982; Stevens et al., 1989) or the human phenylethanolamine-N-methyltransferase (PNMT) gene (Baetge et al.,

1988) were inserted into the 5' end of this construct, giving the plasmids MTCHE β and PTCHE β (see maps in Figure 1 and Experimental Procedures for details of their construction). The constructs were separated from plasmid sequences and injected into the male pronuclei of fertilized mouse eggs. Typical injections ranged from 300–600 molecules per egg. The injected eggs were placed in the uteri of pseudopregnant mice, and live births were recorded. Tail DNA from the progeny was examined for the presence of the transgene by Southern blotting using the influenza C esterase cDNA as a probe.

The MTCHE β Construct Arrests Development at the 2-Cell Stage

After injection of the MTCHE β construct, very few live mouse births were obtained (2 live births from 194 injected eggs). This was in striking contrast with the PTCHE β construct, which gave 26 live births from 152 injected eggs, and with the general experience of the same operator in the same transgenic mouse facility over the last year (see Table 1). The lack of transgenic mice following injection of MTCHE β could be explained by a lethal effect of the incorporated transgene upon development. However, this cannot explain the lack of nontransgenic mice, which are usually in the majority (see Table 1). We therefore reasoned that the developmental arrest must occur at a very early stage as a consequence of transient expression from the injected MTCHE β construct DNA. Such transient expression of injected sequences driven by the metallothionein promoter has been previously described in the fertilized mouse egg, even in the absence of added heavy metals (Brinster et al., 1982; Stevens et al., 1989). To examine this possibility we carried out the *in vitro* experiment reported in Figure 2. Following injection with buffer or an irrelevant construct (pWPB7), most of the injected embryos progressed to the 4-cell stage, and several developed to the morula stage after 4 days. However, all of the eggs injected with the MTCHE β construct stopped development at the 2-cell stage and showed no further progress for up to 4–5 days of observation when they underwent degeneration. This effect was reproducible in several different experiments utilizing two different preparations of the MTCHE β construct. Of a total of 115 fertilized eggs injected with MTCHE β in seven separate experiments, only 1 egg was observed to progress beyond the 2-cell stage.

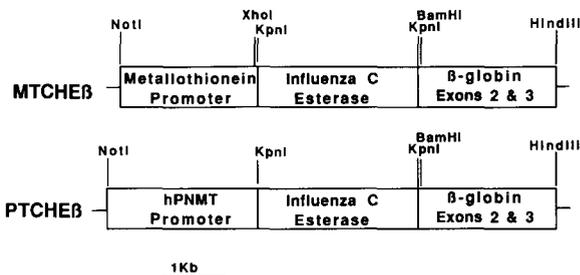


Figure 1. Maps of DNA Constructs Used in this Study

Table 1. Differing Yields of Live Mice with Injection of Different Linearized DNA Constructs into Fertilized Mouse Eggs

Construct Injected	Number of Eggs Implanted	Number of Live Births	Number of Embryos	
			Nontransgenic	Transgenic
MTCHE β	194	2 (1%)	2	0
PTCHE β	152	26 (17%)	18	8
All others (1989-90)	847	108 (13%)	82	26

Fertilized mouse eggs were microinjected with 1-2 pl of linearized DNA constructs at a concentration of 1-2 μ g/ml (300-600 copies per cell) in 5 mM Tris-HCl, 0.1 mM EDTA (pH 7.5). Eggs that survived injection were implanted into the uteri of pseudopregnant females. Live births were recorded, and tail DNA was analyzed for the incorporation of the relevant transgene by Southern blotting.

To rule out a "toxic" effect of the DNA construct itself, the MTCHE β construct was compared with the closely related PTCH β construct, which contains all of the same sequences except for the change in the 5' promoter region (see Figure 1). Injection of similar amounts of the latter construct had no appreciable effect upon the development of the injected mouse eggs in vitro (see Table 2). This is in keeping with the success in obtaining several transgenic mice using the PTCH β construct (see Table 1). Thus, the

Table 2. Effects of Injection of Different DNA Constructs into Fertilized Mouse Eggs

Injection	Number of Eggs	Stage	Number of Embryos			
			Day 2	Day 3	Day 4	Day 5
None	10	Degenerated/abnormal	1	1	3	4
		2-cell	9	4	3	0
		4-cell	0	5	1	1
		>4-cell	0	0	3	5
Buffer	10	Degenerated/abnormal	0	1	2	4
		2-cell	10	1	1	0
		4-cell	0	8	1	0
		>4-cell	0	0	6	6
MTCHE β	12	Degenerated/abnormal	2	2	3	10
		2-cell	10	10	9	2
		4-cell	0	0	0	0
		>4-cell	0	0	0	0
PTCHE β	12	Degenerated/abnormal	1	1	1	3
		2-cell	11	3	2	0
		4-cell	0	8	2	2
		>4-cell	0	0	7	7

Fertilized mouse eggs were microinjected with 1-2 pl of linearized DNA constructs at a concentration of 1-2 μ g/ml (300-600 copies per cell) in 5 mM Tris-HCl, 0.1 mM EDTA (pH 7.5). Groups of eggs that survived the injection were observed for development in vitro in M16 medium.

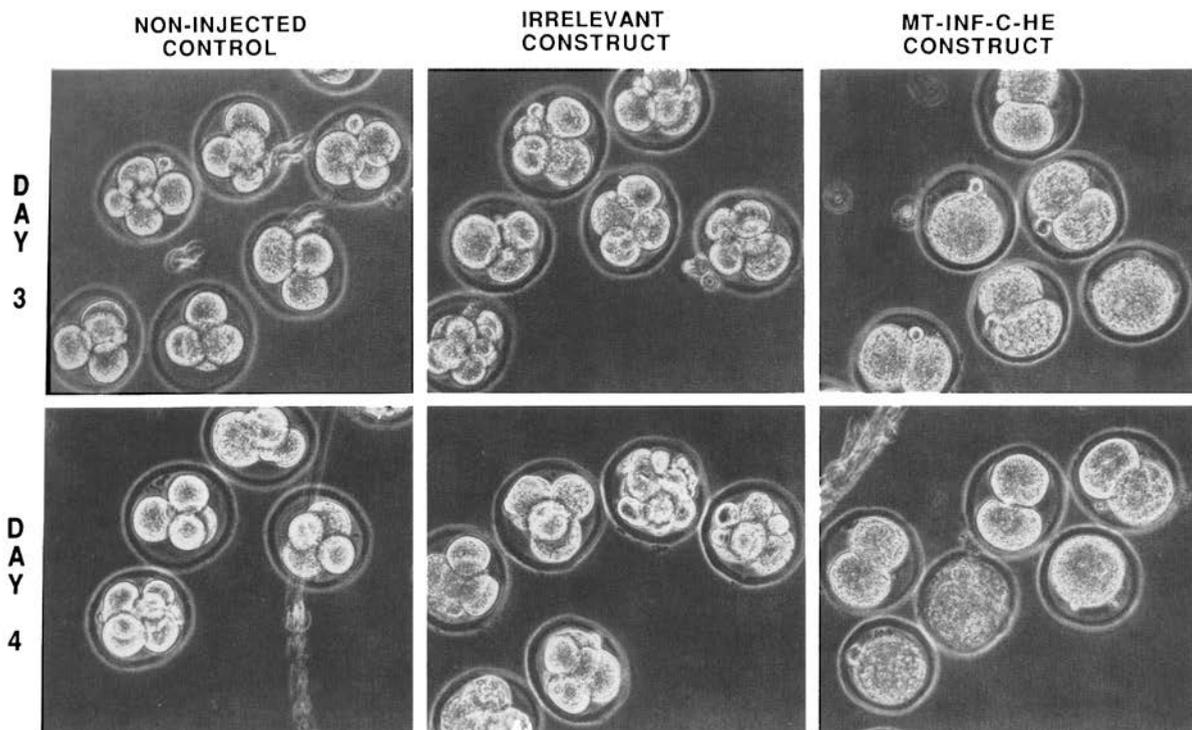
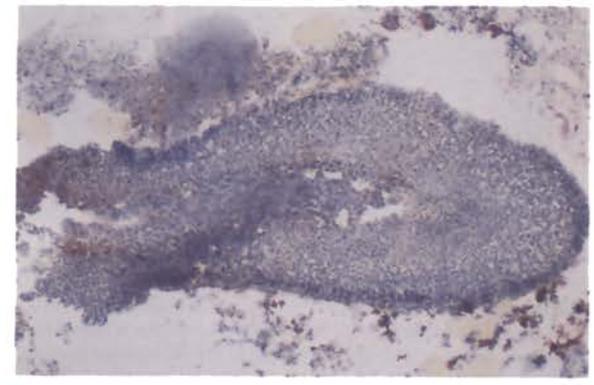
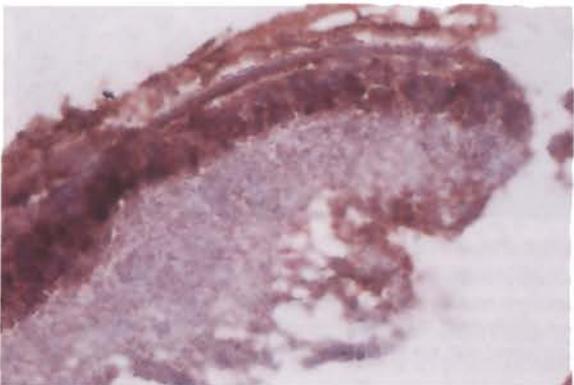
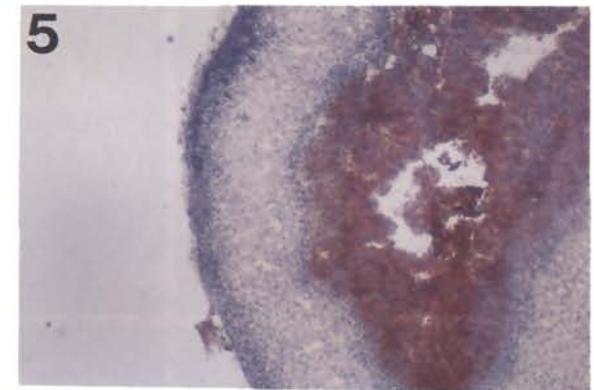
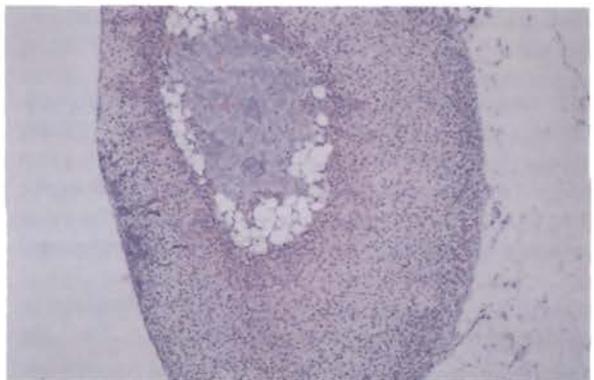
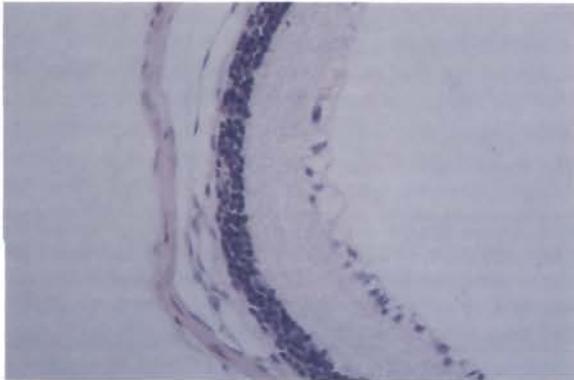
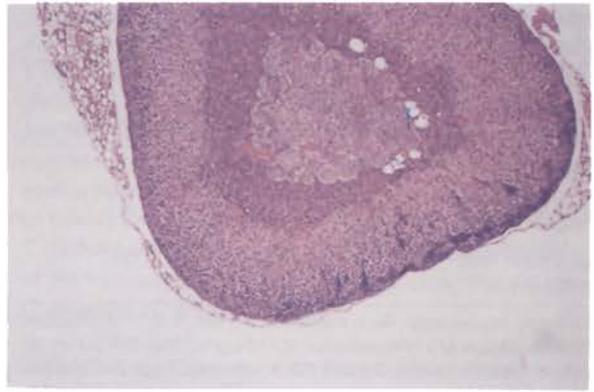
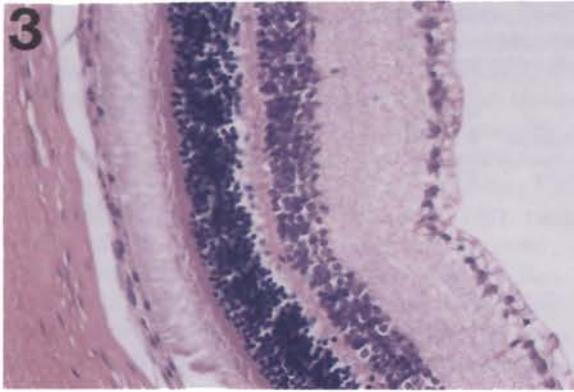


Figure 2. Microinjection of the MTCHE β Construct Causes Developmental Arrest at the 2-Cell Stage

Fertilized mouse eggs were microinjected with 1-2 pl of linearized DNA constructs at a concentration of 1-2 μ g/ml (300-600 copies per cell) in 5 mM Tris-HCl, 0.1 mM EDTA (pH 7.5). Groups of eggs that survived the injection were observed daily for development in vitro in M16 medium. The figure shows the appearance of representative embryos at days 3 and 4. The eggs injected with the irrelevant construct (pWPB7) progressed through to the morula stage in a manner similar to the noninjected control. The eggs injected with the MTCHE β construct (labeled MT-INF-C-HE) were uniformly arrested at the 2-cell stage and did not progress further.



arrest is not caused by the esterase or β -globin sequences themselves, but is dependent upon control of their expression. It is also relevant to point out that many other constructs utilizing the metallothionein promoter (including some encoding surface glycoproteins) have been used successfully by other investigators to obtain transgenic mice (Palmiter et al., 1983; Iwakura et al., 1988; Hofmann et al., 1988; Morahan et al., 1989).

These results suggest that the effects of the MTCHE β construct are mediated by the transient expression of the esterase from the injected DNA. Immunofluorescence studies did not consistently detect surface expression of the esterase protein, presumably because the amount expressed was too low (data not shown). However, catalytic amounts of cell-surface enzyme should be sufficient to destroy O-acetylation and might not be detectable with the antibody.

Expression of the Influenza C Esterase in Selected Tissues of Transgenic Mice Causes Developmental Abnormalities

In contrast with the MTCHE β construct, injection of the PTCHE β construct gave significant numbers of transgenic mice (see Table 1). Such mice appeared grossly normal. Examination of founder mice showed no significant gross or histological abnormalities in a variety of tissues (see Experimental Procedures for list), with the exception of the retina and the adrenals, the two predicted target organs for PNMT promoter-driven expression (Baetge et al., 1988). As expected, some mice with an incorporated transgene showed no expression of the protein and no obvious phenotype. Others showed expression of the influenza C protein in the retina and the adrenal gland and a variable phenotype. In these animals the adrenal glands were very difficult to find. Instead of the normal yellow

button of tissue above the kidney, there was a mass of fat with no obvious or distinct gland. Sectioning through this fat indicated that the adrenal gland was present, and histological examination showed a significant abnormality (Figure 3). The normal adult adrenal gland has three distinct layers in the cortex (zona glomerulosa, zona fasciculata, and zona reticulosa) and a central medulla. In the abnormal gland, the peripheral zona glomerulosa and the central medullary region appeared relatively normal. The zona fasciculata of the cortex appeared somewhat disorganized, with loss of the orderly columns seen in the normal gland. The zona reticulosa (that is normally immediately adjacent to the medulla) was the most abnormal and was almost completely replaced by fatty tissue. The retina was also grossly abnormal (Figure 3). The typical nine layers of the retina were difficult to distinguish and appeared to have been partly fused together with probable photoreceptor atrophy. Of the remaining layers, one resembled a ganglion layer, one a plexiform layer, one a nuclear layer, and one an epithelial layer. However, further studies with cell type-specific and layer-specific antibodies will be required to identify the precise nature of the surviving layers.

Staining of the abnormal retinæ and adrenal glands with antibodies proved to be technically difficult because of the surprisingly fragile nature of the tissues compared with their normal counterparts. However, a polyclonal antibody against the influenza C esterase showed significant expression of the protein in the single nuclear layer of the abnormal retina (see Figure 4). The adrenal gland also showed expression of the protein, although some nonspecific background staining was obtained with the gland from the control mouse (data not shown). Specific staining with the antibody was not seen in any of the other tissues examined, with the possible exception of the kidney tubular cells, which showed a weak reaction.

Figure 3. Abnormalities in the Retina and Adrenal Glands of Mice Transgenic for the PTCHE β Construct

Different tissues from mice transgenic for the PTCHE β construct were examined by hematoxylin and eosin staining of paraffin-embedded sections and compared with normal littermates as described in Experimental Procedures. All tissues examined appeared grossly normal, except for the retina and adrenal gland. The figure compares the retinæ (left panels) and the adrenal glands (right panels) from a normal (upper panels) and a transgenic (lower panels) animal. Magnification is 260 \times for left panels and 65 \times for right panels.

Figure 4. Expression of the Influenza C Esterase Protein in Tissues of Transgenic Mice

Frozen sections of tissues from normal and PTCHE β transgenic mice were evaluated by immunoperoxidase staining with a polyclonal monospecific antibody against the influenza C esterase protein (antibody dilution 1:200). Specific staining was seen only in the retinæ and adrenal glands of the transgenic mice. The figure compares staining of the retinæ from a normal (upper panel) mouse and from a transgenic (lower panel) mouse. Because of the very fragile nature of the transgenic retina, some fragmentation of the tissue during processing was unavoidable. The single nuclear layer of the abnormal retina stains heavily with the antibody. Magnification is 248 \times .

Figure 5. Loss of Expression of 9-O-Acetyl-G₀₃ in the Adrenal Glands of Mice Transgenic for the PTCHE β Construct

Frozen sections of adrenal glands from normal and PTCHE β transgenic mice were evaluated by immunoperoxidase staining with 27A, a monoclonal antibody specific for 9-O-acetyl-G₀₃. Staining of the central medulla is intense in the gland from the normal animal (upper panel, ascites dilution 1:200). Reactivity is much reduced in the gland from the transgenic animal (lower panel) even at an ascites dilution of 1:100. Again, some fragmentation of the abnormal gland appeared to be unavoidable. Magnification is 62 \times .

The establishment of stable breeding lines of mice with these abnormalities proved to be very difficult. Of 40 live progeny obtained by breeding 5 of these founder mice (both male and female), only 2 carried the transgene. We are not sure of the reason for the extremely low penetrance of the transgene in the face of apparently normal fertility of the founders (see below for further discussion). However, for the moment, it makes it very difficult to study the inheritance of these abnormalities and the variability of the phenotype within a single line.

Expression of the Esterase Causes Loss of O-Acetylation of Sialic Acids

A basic assumption of this experiment is that the enzyme will cause loss of 9-O-acetylation of sialic acids on all glycoconjugates in tissues in which it is expressed. Direct chemical demonstration of this outcome is somewhat difficult in complex tissue samples with multiple cell types. However, the ganglioside 9-O-acetyl-G_{D3} is a potential target molecule for the expressed esterase and can be detected by specific monoclonal antibodies (Levine et al., 1984; Sparrow and Barnstable, 1988; J. Reiviren, H. Holthofer, and A. Miettinen, unpublished data). In the murine retina this molecule is expressed prenatally but disappears rapidly in the postnatal period (Sparrow and Barnstable, 1988; Levine et al., 1986). On the other hand, the adrenal gland shows persistent expression of this molecule after birth. We therefore examined the adrenal glands of these mice with the monoclonal antibody 27A, which is specific for 9-O-acetyl-G_{D3} (J. Reiviren, H. Holthofer, and A. Miettinen, unpublished data). As shown in Figure 5, the normal adrenal gland demonstrated a strong staining of the medulla with this antibody, indicating the presence of the 9-O-acetylated ganglioside. In contrast, the medulla from a transgenic adrenal gland showed markedly diminished staining with this antibody. This indicates that expression of the influenza C esterase had the desired effect, i.e., the destruction of the 9-O-acetyl group, which is a required epitope for the binding of this antibody.

In contrast, the glomeruli of the kidney were found to be equally positive for 9-O-acetyl-G_{D3}, both in the normal and in the transgenic animals (data not shown). This suggests that the viral esterase activity is confined to the adrenal gland and the retina and that a circulating active form of the enzyme is not released from these tissues to affect distant sites.

Discussion

In this study, expression of a viral enzyme specific for cleavage of 9-O-acetyl esters on sialic acids caused significant abnormalities in murine embryogenesis. As discussed above, the only known action of this membrane protein under the conditions used is its 9-O-acylesterase activity. Prior work has shown that the expression of such viral receptor-destroying enzymes in cells does result in removal of their cognate carbohydrate structures from the same cell surface (Morrison and McGinnes, 1989; Els et al., 1989). Thus, the early developmental arrest seen with the MTCHE β construct is likely due to removal of 9-O-

acetyl esters from sialic acids from unknown glycoconjugates on the surface of the fertilized egg. The mechanism of arrest is unknown, but implies specific recognition of 9-O-acetyl esters by a lectin-like molecule, which in turn is required to signal further cell divisions. Further investigation of this finding will include searching for such a lectin and delineation of the exact stage of the developmental block. After many attempts, we did finally obtain one mouse transgenic for the MTCHE β construct (see Table 1). We presume that this animal "escaped" the early developmental block, perhaps because that particular embryo happened to get a lower dose of injected DNA. This female, which appears normal, is now being carefully analyzed to see if it is expressing the transgene.

Examination of the mice transgenic for the PTCHE β construct revealed interesting abnormalities in the retina and the adrenal gland, while all the other tissues were essentially normal. Thus, the only two tissues that were obviously affected were those in which the PNMT promoter is known to be active in late embryogenesis (Baetge et al., 1988). This provides reassurance that the phenotype seen is not due to random disruption of an unrelated endogenous gene by the transgenic insert. Furthermore, monospecific antibodies demonstrated expression of the viral protein in the retina and loss of 9-O-acetylation in the adrenal gland. In contrast, O-acetylation was unaffected in the renal glomeruli, where the enzyme was not expressed.

The degree of abnormality in the target organs varied between different mice, perhaps because of the effect of transgene position upon the precise timing and/or level of expression of the enzyme in the target tissues. In the most severe situation, the retina showed gross disorganization with loss of many normal layers. The appearance suggests degeneration of the photoreceptor layer and fusion and/or failure of formation of some of the other layers. It is tempting to speculate that this phenotype was caused by disruption of the previously reported dorsal-ventral gradient of 9-O-acetyl-G_{D3} across the embryonic retina (Constantine-Paton et al., 1986; Sparrow and Barnstable, 1988), which could be playing a role in the normal organization of the retinal layers. Alternatively, O-acetyl groups could have been removed from some other important glycoconjugate. Further detailed analysis of such altered retinæ at different stages of development will be required to define the primary abnormality and to identify the nature of the surviving cells and layers.

The abnormalities seen in the adrenal gland are also compatible with prior knowledge concerning the development of this organ (Waring, 1936; Bech et al., 1969; Theiler and Muntener, 1974; Seron-Ferre and Jaffe, 1981). During normal embryogenesis, the medullary cells migrate from the neural crest and invade the developing cortex, which is derived from mesenchyme. The two types of cells are initially mixed together, but eventually separate. At birth this separation is incomplete, and a zone of mixed cells exists between the two components. This is precisely the region of the gland that shows the most obvious abnormality in the transgenic animal. Again, it is tempting to speculate that loss of O-acetylated G_{D3} altered cell-cell interactions critical for complete separation of the medulla and

cortex. Alternatively, local–regional hormonal interactions that are known to occur between the cortex and the medulla could have been disrupted by abnormal functioning of the medullary cells. Exploration of such hypotheses requires the detailed analyses of homozygous mice with these phenotypes at different stages of embryonic development. This would also allow more careful analysis of the regions of the brain known to express PNMT-positive neurons (Baetge et al., 1988).

However, attempts to establish such a homozygous line of mice have proven difficult because of a markedly low frequency of transmission of the transgene. It is possible that the expression of the transgene after the first generation results in selective gamete loss or fetal loss. For example, it is possible that more promiscuous expression of the transgene might occur in the F1 generation, resulting in the block at the 2-cell stage seen with the MTCHE β construct. These and other possibilities need to be investigated further.

It is now commonplace to use tissue culture cells to study a highly conserved gene or gene product that is not required for the growth of the cells, but whose functions in the intact multicellular organism are essentially unknown. Many oligosaccharide structures fall into this category. In this report, we have described a novel approach to exploring the biological roles of such oligosaccharides during development. Rather than interfering with the genetic and cellular machinery responsible for the synthesis of these molecules, we have chosen to eliminate them by expressing a specific degradative enzyme as a cell-surface molecule. This also avoids the possibility of nonspecific interference with the normal functioning of the Golgi–ER pathway (Farquhar, 1991) that might occur with deletion of a biosynthetic enzyme. The use of specific promoters allows the analysis of tissue-specific functions of oligosaccharides in later aspects of development, sidestepping the possibility of an early lethal event that could have occurred with a gene “knockout” experiment. This approach is also superior to that of expressing multivalent carbohydrate-specific lectins or antibodies, which could cause nonspecific intercellular adhesion. In principle, it could be generalized to any situation in which a cDNA is available encoding a specific oligosaccharide-degrading enzyme in a membrane-bound form.

Experimental Procedures

DNA Clones and Cloning Methodology

A 2.2 kb BamHI–PstI fragment containing the second and third exons of the human β -globin gene (including signals for polyadenylation) (Treisman et al., 1983) was subcloned into the polylinker site of the commercial vector PKS (Stratagene). The fragment was re-excised with BamHI–HindIII digestion and inserted into the polylinker region of the pBR322-based vector pDPL.13 (Gendel et al., 1983) (kindly provided by Steven Gendel, University of Iowa). The NarI site at the 5' end of this plasmid was converted into a NotI site to give pDPP β (NotI). Sequences encoding the influenza C esterase from InfC/Cal/78 were derived from the plasmid pCHE4 (Vlasak et al., 1987) (kindly provided by Dr. Peter Palese, Mount Sinai School of Medicine). The HpaI site at the 3' end was converted into a KpnI site, and the 2.2 kb KpnI fragment containing the coding sequences was inserted into the KpnI site of pDPP β (NotI) to give pDPCHE β . Two different promoters were

inserted into the 5' end of this construct. The murine metallothionein promoter region was obtained from plasmid pMGH (Palmiter et al., 1983) (kindly provided by M. G. Rosenfeld, University of California, San Diego). The EcoRI site of this 1.8 kb region was converted into a NotI site, and the NotI–XhoI fragment was excised and inserted into pDPCHE β to give MTCHE β . The 2 kb promoter region from the 5' end of the human PNMT gene (Baetge et al., 1988) was kindly provided by Richard Palmiter (University of Washington). The EcoRI site at the 5' end was converted into a NotI site, and the BamHI site at the 3' end was converted into a KpnI site. The NotI–KpnI fragment was excised and inserted into pDPCHE β to give PTCHE β . The two final constructs were linearized and freed of vector sequences by digestion with NotI and HindIII (see Figure 1) and purified on sucrose gradients as previously described (Dent et al., 1990).

Antibodies and Immunological Reagents

A rabbit polyclonal monospecific antibody against the influenza C esterase protein was kindly provided by Peter Palese (Mount Sinai School of Medicine). The murine monoclonal antibody 27A (Dekan et al., 1990; J. Reiviren, H. Holthofer, and A. Miettinen, unpublished data), known to be specific for 9-O-acetylated gangliosides, was kindly provided by Marilyn Farquhar (University of California, San Diego). All secondary antibodies, developing reagents, and staining reagents were obtained from commercial sources.

Mice

All experiments described were done with pathogen-free (C57BL/6J \times SJL/J)F₁ mice obtained from Jackson Laboratories. Mice were maintained in a constant temperature and humidity environment and a 10–14 hr light/dark cycle. Food and water were provided ad libitum.

Microinjection of Eggs and Growth In Vitro

Culture and microinjection of fertilized oocytes was carried out as described (Hogan et al., 1986). Briefly, 10-week-old female mice were superovulated using 5 IU of pregnant mare serum followed 48 hr later by 5 IU of human chorionic gonadotropin and immediately mated with males. The next morning females with plugs were sacrificed, and the eggs were washed from the dissected oviduct. Eggs were washed in M2 medium and transferred to M16 medium for culture at 37°C (5% CO₂, 6% O₂, 89% N₂) (Monk, 1987). Eggs were injected with DNA constructs in M2 medium and cultured in M16 medium. Typical injections ranged from 1–2 pl per egg, at a DNA concentration of 1–2 mg/ml (300–600 molecules injected per egg).

Analysis of Normal and Transgenic Mice

Tail DNA was extracted as previously described (Hogan et al., 1986), digested with EcoRI, and analyzed by Southern blotting using a ³²P-labeled 2.2 kb fragment encoding the influenza C esterase gene, free of murine or human sequences. Transgenic mice and normal littermates were anesthetized, exsanguinated, and sacrificed for examination of several different tissues (brain, eye, skeletal muscle, heart, lung, liver, spleen, pancreas, kidney, adrenal gland, and small intestine). Organs were removed, and pieces were placed either in 10% buffered formalin for routine histological analysis or in 15% buffered sucrose at 4°C prior to flash freezing in isopentane at liquid nitrogen temperature. For standard histological analyses, the formalin-fixed specimens were routinely processed, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. For immunohistochemical analyses, 5 μ m frozen sections were cut and mounted onto gelatin-coated slides, air dried briefly, fixed for 10 min in 10% buffered formalin, and then overlaid with 10% goat serum in 1% bovine serum albumin, phosphate-buffered saline (pH 7.0) for 20 min. Appropriate dilutions of rabbit antibody to influenza C esterase were then overlaid on the sections and allowed to incubate at 4°C overnight or at 37°C for 2 hr in a humid chamber. The slides were washed in phosphate-buffered saline and overlaid with appropriate dilutions of peroxidase-labeled goat anti-rabbit antibody (Bio-Rad Laboratories, Richmond, CA) for 30 min at room temperature. The slides were washed again and substrate solution, consisting of 3',5'-aminoethylcarbazole in 0.1 M sodium acetate and 0.03% H₂O₂, was overlaid for 15 min. The sections were washed, counterstained with hematoxylin, washed again, mounted using glycerol gelatin (Sigma, St. Louis, MO), and viewed.

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