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# Glycoprotein Metabolism in Skin Cancer: Synthesis, Pool Size, and Partial Characterization of Glycoproteins in the Rat Basosquamous Cell Carcinoma

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**Uptake of fucose, glucosamine, galactose, and mannose and the incorporation of these sugars into glycoconjugates have been quantified in a model epithelial tumor, the basosquamous cell carcinoma of the rat. Following isolation of glycoprotein and papain digestion, the fucosylated glycopeptides were fractionated according to molecular weight ($M_r$) and by affinity chromatography.**

**Analysis of cellular material revealed a 2- to 3-fold reduction in the proportion of high-$M_r$ fucopeptides synthesized by the tumor compared with normal epidermis, accompanied by a profound block in the incorporation of galactose into glycoconjugates. Parallel investigation of carbohydrate removed from the cell surface with trypsin or spontaneously shed into the medium indicated a striking decrease in the total release of fucopeptides from the tumor; the $M_r$ was (respectively) normal or increased. Thus the fucopeptide abnormality appears to be accounted for almost entirely by a shift toward accumulation of low-$M_r$ material at internal locations within the cell.**

A number of transformation-specific alterations in glycoprotein metabolism have been reported, mostly in cells of nonepithelial origin [1–3]. These are of considerable interest because cell surface glycoprotein may be a major determinant in cell behavior [4] and subtle changes in glycoprotein composition may result in profound disturbance of homeostatic mechanisms regulating differentiation and growth control [5].

Unfortunately, little information is so far available concerning malignancies of epithelial origin. Here we present data regarding glycoprotein synthesis, pool sizes, and composition in a model tumor of this kind, in an effort to establish how far such changes may be general features of malignancy.

## MATERIALS AND METHODS

### *Material*

The WAG/Rij rat skin basosquamous cell carcinoma was selected for this study because it is relatively easy to maintain, is stable on repeated passaging, and its morphologic and growth characteristics are well documented [6]. A specimen of this tumor was obtained from the Radiobiological Institute TNO, Rijswijk (Prof. Dr. G. W. Barendsen) and was transplanted by i.p. injection of pieces of tumor of about 1 $mm^3$ into WAG/Rij rats. The cellular DNA content of our material was 3.4 *n* (personal communication, Dr. F. W. Bauer, Dept. of Dermatology, University of Nijmegen). Skin from healthy WAG/Rij rats was used as control.

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Abbreviations:
TCA: trichloroacetic acid

### *General Methods*

The methods used have been reported previously and are summarized in Fig 1. Briefly, cells were isolated from keratotome slices by trypsinization in the presence of dithioerythritol [7] and suspended in TC199 medium supplemented with 20% calf serum. Radioactive sugars (1 $\mu$Ci/ml for $^{14}$C-labeled sugars or 10 $\mu$Ci/ml for $^{3}$H-labeled sugars) were added and the suspension was incubated at 37°C according to three time schemes [8]: (1) a single labeling period of 3 h (t = 3); (2) a single labeling period of 22 h (t = 22); (3) preincubation in TC199 medium plus serum for 22 h followed by a labeling period of 3 h (t = 22/3).

Total cellular labeled glycoconjugates were isolated by trichloroacetic acid (TCA) extraction [8]. The "TCA-insoluble sugar" was further fractionated [9] into a "lipid fraction" (chloroform-methanol extraction) and a "glycosaminoglycan and glycopeptide fraction" (papain digestion of chloroform-methanol extracted residue). Determination of the shedding of labeled glycoconjugates into the medium and isolation of cell surface glycopeptides, released by trypsin, from cells labeled for 22 h were identical to those previously reported [10]. Glycopeptides were analyzed by gel filtration on Sephadex G-50 or by affinity chromatography on concanavalin A-Sepharose 4B as described earlier [11]. Some experiments were carried out using pronase in place of papain for glycoprotein digestion; the elution pattern of the oligosaccharides following gel filtration was essentially unchanged.

The theoretical basis for our selection of labeling times and for our interpretation of data has already been presented [8]. In brief, uptake and incorporation of sugars by keratinocytes is linear for several hours and the "short" experiments (t = 3) may be used to evaluate rates of synthesis. Equilibrium labeling (similar specific activities for all pools) is established by 22 h and the ratio of individual fractions at this time indicates their relative pool size. Preincubation experiments (t = 22/3) are required to verify whether metabolic parameters remain constant during the equilibration period.

## RESULTS

### *Uptake and Incorporation of Sugars*

Data obtained regarding the entry rates of sugars into keratinocytes (radioactivity in TCA-soluble pool at t = 3 and t = 22/3), the rate of synthesis of glycoconjugates (ratio of activity in TCA-insoluble:activity in TCA-soluble pool at t = 3 and t = 22/3), and pool sizes (activity in TCA-soluble and TCA-insoluble pool at t = 22) are given in Fig 2.

It is seen that the carcinoma cells take up all sugars more rapidly from the medium than do normal cells (Fig 2*a*). In contrast to the other sugars, the rate of entry of galactose into normal and carcinoma cells declines considerably after 22 h preincubation in vitro (t = 22/3). For all sugars, except galactose, the radioactivity in the TCA-soluble pool of both types of cells at t = 22 is equal to the level found at t = 3 and appears to be higher for carcinoma in comparison with normal cells. The pool sizes for TCA-insoluble glycoconjugates of carcinoma cells (t = 22) are increased over those from normal cells, especially when mannose is used as precursor.

The rate of synthesis of glycoconjugates (Fig 2*b*) using galactose as precursor appears to be strikingly lower for carcinoma cells in comparison with normal cells (Wilcoxon ranking test, $p < 0.01$ for t = 3 and t = 22/3). For glucosamine and mannose, however, a higher rate of incorporation has been found for carcinoma cells compared to normal cells (Wilcoxon ranking test, $p < 0.01$ and $p = 0.01$, respectively, for t = 3).

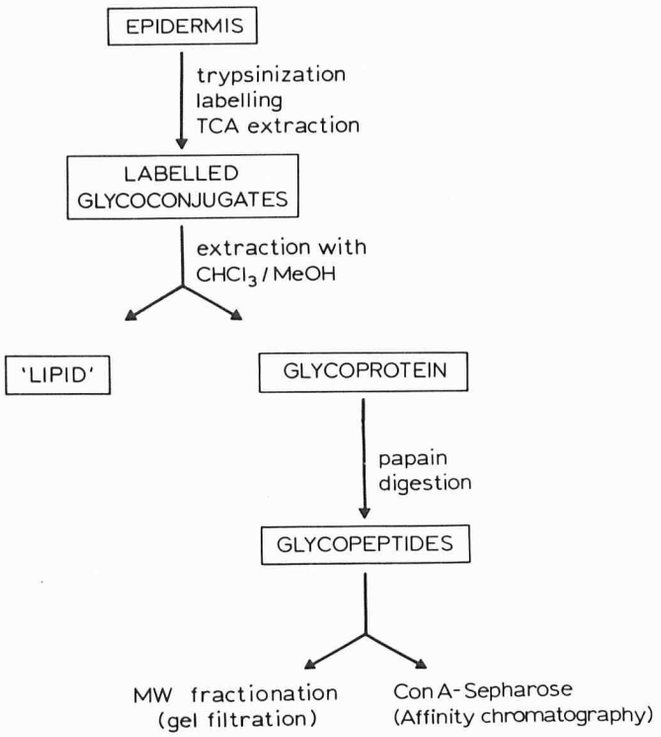


FIG 1. General protocol for isolation of the various fractions.

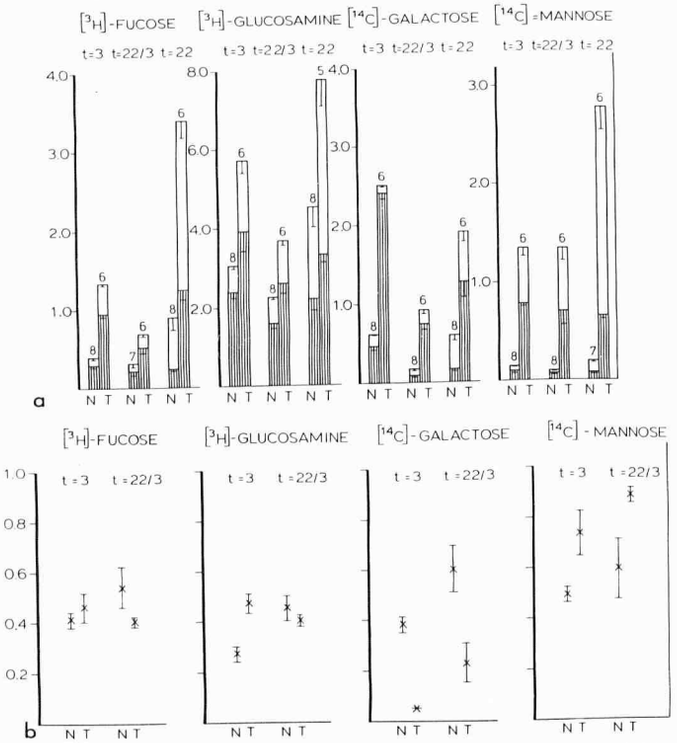


FIG 2. *a*, Radioactivity (cpm per $10^3$ cells) in the TCA-soluble fractions (*shaded bars*) and the TCA-insoluble fractions (*open bars*) of rat carcinomas and controls. The *figure above each bar* indicates the number of specimens measured, and the *vertical lines* represent the SEM. *N* = normal epidermis, *T* = tumor. *b*, Ratios of TCA-insoluble to TCA-soluble activity.

*Composition of Total Cellular Glycoconjugates*

The TCA-insoluble material from the t = 22 experiments was extracted with chloroform-methanol to yield a "lipid fraction." It is seen from Table I that, regardless of the sugar employed, the percentage of radioactivity in this fraction was the same for tumors and controls. The lipids were not analyzed further.

The residual material was digested with papain and subjected to gel filtration on Sephadex G-50. Elution profiles (using [$^3$H] fucose) are illustrated in Fig 3; it is seen that malignancy is accompanied by a substantial shift to lower $M_r$. For convenience of quantification, 4 regions (A–D) have been designated of decreasing $M_r$ [9]. The percentage distributions of radioactivity, using the 4 different sugars, are summarized in Table II. It is seen that glucosamine labeling gives a picture similar to fucose labeling, namely a relative decrease in fraction B and an increase in fraction C in the tumor compared with normal skin. Using either galactose or mannose as label, no significant change in the C/B ratio was observed.

In some experiments an aliquot of the papain digest was chromatographed on concanavalin A-Sepharose 4B, which binds mainly N-glycosidic glycopeptides of the complex type with 2 chains. An increase in biantennary material was found to accompany malignancy, the normal value of 37 ± 3% (6 experiments) rising to 46 ± 2% (3 experiments) in the tumors.

*Shed and Cell-Surface Glycoconjugates*

It was clearly of interest to establish how far the total cellular fucopeptides (above) were representative for the individual metabolic pools. Two of these were investigated: first, the material spontaneously "shed" into the medium (secreted glycoprotein plus bound surface material), and second, the cell-surface glycoprotein removed by exposure to trypsin.

Remarkably, the spontaneous shedding of glycoprotein by malignant cells was very much lower than that of normal cells; calculated as percentages of total fucose-labeled glycoproteins, the shed material amounted to 44 ± 6% and 9 ± 3% (mean ± SEM, 4 and 6 experiments each) for normal and carcinoma cells, respectively. The $M_r$ distributions of the fucopeptides obtained by papain digestion of the shed material are given in Table III. It is seen that the changes here are in the opposite direction to those described for the total cellular material, in this case a decreased C/B ratio for the tumor.

TABLE I. *Percentages of lipid-soluble radioactivity (mean ± SEM) using various precursors*

| Precursor | Normal | Tumor |
|---|---|---|
| [$^3$H]Fucose | 28 ± 3 (11)[a] | 27 ± 2 (19) |
| [$^3$H]Glucosamine | 40 ± 4 (4) | 36 ± 2 (12) |
| [$^{14}$C]Galactose | 54 ± 3 (3) | 55 ± 2 (8) |
| [$^{14}$C]Mannose | 50 ± 1 (3) | 55 ± 6 (6) |

[a] Numbers of specimens are shown in parentheses.

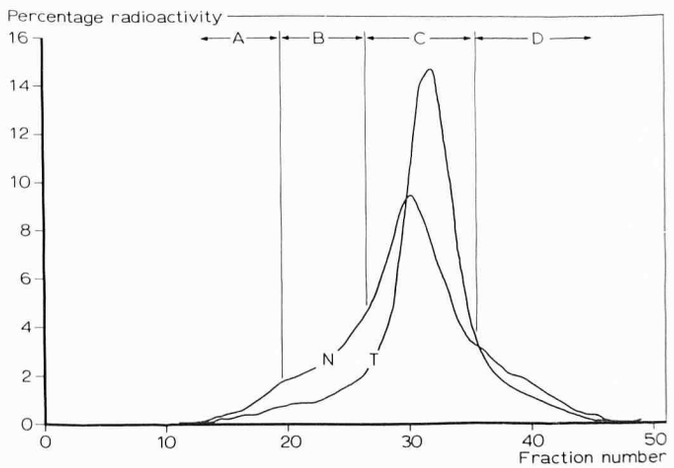


FIG 3. Elution profiles of cellular fucopeptides on Sephadex G-50 from carcinomas (*T*) and normal epidermis (*N*). Following labeling for 22 h with [$^3$H]fucose, extraction of glycoprotein and digestion with papain was as described in the text. Curves were averaged from 9 specimens (*T*) and 6 specimens (*N*).

TABLE II. *Distribution of glycopeptides from cellular digests into fractions of decreasing $M_r$*

| Sugar | Specimen | Fraction | | | | Ratio C/B |
|---|---|---|---|---|---|---|
| | | A | B | C | D | |
| Fucose | Normal (6) | 4.3 ± 0.2 | 20.8 ± 1.4 | 57.5 ± 2.0 | 17.2 ± 0.9 | 2.8 ± 0.6 |
| | Tumor (9) | 2.6 ± 0.3 | 8.9 ± 0.6 | 76.8 ± 1.2 | 11.5 ± 1.2 | 9.1 ± 0.8 |
| Glucosamine | Normal (3) | 7.6 ± 1.0 | 17.6 ± 1.2 | 39.3 ± 2.6 | 35.4 ± 2.5 | 2.2 ± 0.1 |
| | Tumor (9) | 7.6 ± 0.9 | 11.4 ± 1.6 | 55.3 ± 1.0 | 25.6 ± 2.9 | 6.2 ± 1.3 |
| Galactose | Normal (3) | 8.9 ± 3.9 | 11.4 ± 0.5 | 32.9 ± 1.0 | 46.5 ± 2.4 | 2.8 ± 0.1 |
| | Tumor (10) | 20.8 ± 4.4 | 17.5 ± 1.1 | 35.4 ± 2.0 | 26.4 ± 2.5 | 2.1 ± 0.2 |
| Mannose | Normal (3) | 6.5 ± 0.3 | 9.1 ± 1.3 | 27.1 ± 1.0 | 57.3 ± 1.9 | 3.1 ± 0.4 |
| | Tumor (7) | 5.0 ± 1.2 | 9.6 ± 1.0 | 31.6 ± 3.0 | 53.8 ± 4.7 | 3.4 ± 0.3 |

[a] Figures are percentages (mean ± SEM); numbers of specimens are in parentheses.

TABLE III. *Distribution of glycopeptides from shed and trypsin-released material*

| Parameter | Specimen | Fraction | | | C/B |
|---|---|---|---|---|---|
| | | A | B | C | |
| Shedding | Normal (4) | 0.6 ± 0.2 | 9.1 ± 0.2 | 90.3 ± 0.3 | 9.9 ± 0.3 |
| | Tumor (6) | 2.4 ± 0.4 | 15.1 ± 1.1 | 82.6 ± 0.5 | 5.7 ± 0.6 |
| Trypsinate | Normal (4) | 3.0 ± 0.4 | 17.4 ± 1.8 | 79.5 ± 2.2 | 4.8 ± 0.7 |
| | Tumor (7) | 4.6 ± 0.4 | 15.6 ± 1.2 | 79.9 ± 1.6 | 5.4 ± 0.5 |

[$^3$H]Fucose was used as precursor; figures as for Table II.

Trypsinization also suggested a reduced availability of glycoprotein on the cell surface, twice the amount of material being released by trypsin from control cells compared to carcinoma cells (16 ± 1% and 8 ± 1%, percentages of total fucose-labeled glycoproteins ± SEM, 4 and 7 experiments each). The $M_r$ distributions of fucopeptides obtained by papain digestion of the trypsin-released material appear to be similar (Table III).

## DISCUSSION

It is clear from these results that the metabolism of glycoproteins is grossly disturbed in the basosquamous cell carcinoma of the rat. Entry rates of sugars into the cell, rates of incorporation of these sugars into glycoconjugates, and the pool sizes of both TCA-soluble and conjugated sugars are all, in general, increased; the $M_r$ profiles of fucopeptides are altered, and in addition we see marked changes in the distribution of glycoprotein between the interior of the cell and the cell surface.

Although it is difficult to give a mechanistic explanation which will unify these observations, we may perhaps center our speculation around the one striking exception to the generally increased incorporation rates, namely galactose (Fig 2*b*, t = 3). Tumor cells seem able to transfer this sugar to glycoconjugates at less than one-fifth the rate of normal epidermis. Thus it is tempting to suggest a block in the synthesis of glycoproteins at the point of addition of galactose, a concept which is compatible with the general shift to lower $M_r$ of the glycopeptide fragments. It is also in line with the gross reduction in total glycoproteins on (or shed from) the cell surface, since transport of these substances to the various cell compartments is directed by specific "markers" which in this case would seem to be absent. It may be noted that the theoretical possibility of metabolic conversion of galactose to other sugars (which cannot be excluded) would, by providing a by-pass route, minimize rather than exaggerate the observed differences between the tumor and normal epidermis.

Other explanations that seem unlikely include the following. A preferential release of differentiated cells from normal skin by trypsinization could, in theory, account for the observed changes; this is excluded since microscopic examination verified that very few basal cells remain adherent to the dermis following our procedure [7]. In the case of carcinoma the possibility of contamination with infiltrating cells cannot be entirely ruled out, but these would in any case represent an extremely small percentage of the total population. It remains a theoretical possibility that, due to cell surface alterations, the accessibility of glycoprotein to trypsin is reduced in the malignant cells. This point would require analysis of isolated membrane preparations to exclude it with certainty.

Comparison with previous data is conveniently divided into quantitative aspects (entry and incorporation rates and pool sizes) and structural considerations. Quantification of sugar uptake and its incorporation into glycoconjugates has, in the past, yielded a rather confused picture. A number of detailed early studies of sugar transport (reviewed in [12]) showed, in agreement with our present finding, an increased entry rate for several sugars following malignant transformation in cells of fibroblastic origin. Analyses of sugar content, however, (i.e., pool sizes) have "failed to show any consistent differences and have provided little insight into changes in the oligosaccharides of membrane proteins" [13]; recent interest in this direction has waned considerably.

By contrast, much literature is now available regarding changes in oligosaccharide structure in malignancy. This field has been well reviewed [5,13]. Our present findings seem to differ from these previous reports in two respects. First, the shift to lower $M_r$ in *cellular* fucopeptides described here has not been reported previously; secondly, the consistently increased $M_r$ of fucopeptides derived from *cell-surface* material (trypsinates) could not be demonstrated in the present study.

It is clearly of importance to ascertain whether these findings for the rat carcinoma are characteristic for epithelial malignancies in general or whether they are simply atypical in this respect. A more extensive survey of epitheliomas is in progress in our laboratories and will be reported in due course.

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