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α_1-acid glycoprotein (AGP): a possible carrier of sialyl lewis X (slewis X) antigen in colorectal carcinoma

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Summary. Objectives: 1- to detect α_1-acid glycoprotein (AGP) and sialyl Lewis x (sLex) in colorectal malignant, benign and normal samples; 2- to isolate AGP from colorectal cancer and 3- to study its immunoreactivity with an anti-sLex monoclonal antibody (MAb). Materials and methods: tissue and serum samples from 88 patients with colorectal cancer, 22 adenomas and 23 normal were included. Expression of AGP and sLex was studied by immunohistochemistry (IHC); isolation approach: AGP was precipitated with ammonium sulphate and immunoprecipitated with anti-AGP MAb. The immune complex formed was isolated by protein A-Sepharose CL-4B affinity chromatography and further eluted; fractions were analysed by SDS-PAGE and Western-blot. Statistical analysis was performed by means of Principal Component Analysis. Results: by Western blot employing anti-AGP MAb and sLex MAbs, isolated fractions from malignant samples showed a band at about 45kD. IHC revealed that AGP was expressed in 70% of colorectal carcinoma samples, 50% of benign and 35% of normals. SLex was detected in 31% of malignant samples, 41% of benign and in one normal sample. In malignant samples, AGP reaction comprised the whole specimen with a strong and homogeneous staining while normal and benign samples showed a restricted reaction. In cancer, sLex expression consisted in an intense reactivity in membrane, cellular debris and some cytoplasmic foci while normal and benign samples were occasionally stained. A statistically significant positive correlation was found between AGP and sLex expression. Serum AGP levels were measured by radial immunodiffusion and statistical comparative analysis with tissue expression did not show a correlation between both parameters. Conclusion: AGP may constitute a carrier of sLex in colorectal cancer.

Key words: α_1-acid glycoprotein (AGP), Sialyl-Lewis x (slex), Colorectal carcinoma, Metastasis

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Introduction

Colorectal cancer constitutes an important public health problem worldwide. Despite significant advances in diagnostic endoscopy and radiology and improvement in surgical techniques, the overall prognosis for colorectal cancer has improved only slightly during the past two decades.

It is generally considered that prognosis is closely related to tumor stage at diagnosis but in many cases, it is not possible to detect micrometastatic disease; first detection is frequently performed at an advanced stage and dissemination is usually found. Different carbohydrate antigens have been implicated in invasion and metastasis of colorectal cancer such as sialyl Lewis x (sLex) (Itzkowitz et al., 1986; Yuan et al., 1987); the relevance of this hapten for metastasis formation found additional support after discovering that sLex is the ligand for E-selectin and P-selectin (Phillips et al., 1990). Colon cancer cells bind via sLex and sLea to E-selectin expressed on human umbilical vein endothelial cells *in vitro* (Takada et al., 1993) and they employ this mechanism to extravasation (Sawada et al., 1994).

In colorectal cancer, diverse putative carriers for sLex have been proposed such as mucins and mucin-like proteins (Matsushita et al., 1990; Hanski et al., 1993, 1995). Hanski et al. (1995) found that glycoproteins separated from colon cancer patient sera contained different components including one of 44 kDa band which reacted with anti-α_1-acid glycoprotein (AGP) polyclonal antibody and also with AM-3, an anti-monomeric sLex monoclonal antibody (MAb).

AGP is one of the acute phase proteins mainly synthesized in liver parenchymal cells and IL-1, IL-6 and glucocorticoids are the major modulators of AGP gene expression in these cells (Baumann et al., 1989; Baumann and Gauldie, 1990). However, it has been shown that lymphocytes, granulocytes and monocytes also express a membrane associated form of AGP (Gahmberg and Anderson, 1978); it has also been reported that monocytes can serve as a source of soluble AGP (Nakamura et al., 1993). Increased serum levels of

AGP has been detected in different conditions including malignancy (Bacchus, 1965; Fournier et al., 2000; Croce et al., 2001). Tamura et al. (1981) found an AGP-protein like in ascitic fluid of cancer patients; furthermore, cancer cells may express AGP and this expression is increased in hepatic metastatic focus (Chandrasekaran et al., 1984).

Although different glycoproteins have been found to be carriers of sLex, results are not conclusive; therefore, we developed this research to: 1- isolate and purify AGP from colorectal cancer tissues; 2- to study its immunoreactivity with anti-sLex MAb and 3- to analyze AGP and sLewis x immune expression in colorectal malignant, benign and normal samples.

Materials and methods

Serum and tumor tissue samples

Tissue and serum samples from 88 patients (63 men and 25 women) with colorectal carcinoma; mean age at diagnosis was 65 years (range 42-82, years) were included. All tumors were classified according to UICC criteria TNM Classification of Malignant Tumors (48% corresponded to Stage II, 33% to Stage III and 19% to Stage IV).

Tissue and serum samples from 22 (15 men and 7 females) patients with adenoma and from 23 normal subjects (17 men and 6 females), age (43-76 years) were also included. Colorectal adenomas were classified according to their histological type (tubular, tubular-villous, villous) and the grade of dysplasia (low and high).

Venous blood was obtained aseptically from patients and controls, allowed to clot at room temperature and centrifuged at 3000xg at 4 °C; sera were separated, aliquoted and frozen at -70 °C. Samples were thawed out only once before used.

Antibodies

An anti sLex MAb, KM93 (IgM) (Hanai et al., 1986), a rabbit anti-human orosomucoid (AGP) polyclonal antibody (Code No. A011, DAKO, USA) and mouse anti-human α1-acid glycoprotein MAb (Sigma-Aldrich, St Louis,MO) were assayed for immunohistochemical analysis as well as immunoblotting assays.

Purification procedure

Purification procedure (Bilrup-Jensen, 2001) (summarized in Fig. 1): it was performed in a total of 29 adenocarcinoma specimens belonging to patients with 40% to stage II, 45% to stage III and finally, 15% to stage IV.

Preparation of homogenates and subcellular fraction

Human tumor tissues were mechanically homogenized with a Politron PT1200 mixer (Kinematica) in a lysis buffer (10 mM TRIS buffer, 1mM PMSF, pH 7.4), at 4 ml/g; homogenates were centrifuged at 600xg and at 105000xg at 4 °C and precipitates containing extranuclear membrane fractions were resuspended in 0,01M Tris. Both precipitates and supernatants were concentrated and stored at -70 °C.

Salting out

Precipitation of unwanted proteins was performed with ammonium sulphate at a concentration of 1.6 mol/I. Ammonium sulphate was weighed into a beaker and then samples (tissue homogenate or subcellular fractions) were added, the mixture was stirred until all of the salt was dissolved. The covered beaker was left at room temperature overnight followed by centrifugation at 2700xg for 40 min. The precipitate was washed 3 times with a washing solution (234.9 g ammonium sulfate in 1L distilled water) and a centrifugation step was carried out between each washing; excess salt was extracted by dialysis. Both precipitate and supernatant were dialyzed against distilled water (for 2 days, with a daily change) and phosphate saline buffer (PBS) 0.01 M (2 days, with a daily change) and finally concentrated.

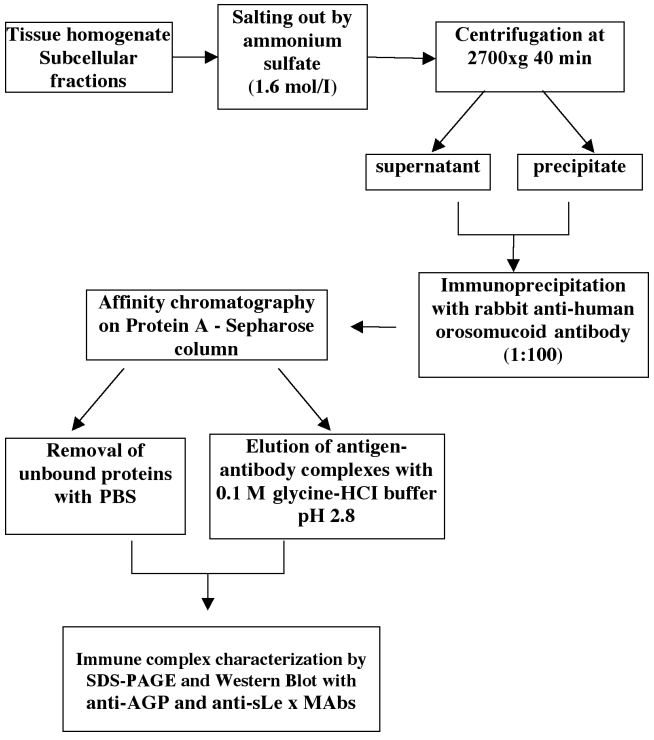

Fig. 1. Schematic summary of methodology employed to isolate and purify AGP from colorectal carcinoma samples.

Immunoprecipitation

Fractions obtained after salting out, dialysis and concentration were precipitated with rabbit anti-human orosomucoid (Dako, Glostrup, Denmark) at a concentration of 1:100; the immune complex (AGP-anti-AGP) was purified with protein A-Sepharose CL-4B beads (Sigma-Aldrich, St Louis, MO) (Gupta et al., 1983) and incubated for an hour at room temperature with gentle rocking. The unbound proteins were removed by washing the solid phase with PBS and the antigen-antibody complex was eluted with 0.1 M glycine-HCI buffer pH 2.8. Collected fractions were dialysed against 0.01 M PBS at 4 °C for 48 h and concentrated.

Immune complex characterization

The proteins of isolated immune complexes were separated and characterized in 10% sodium dodecyl-sulfate polyacrylamide slabs gels (Laemmli, 1970). The samples were suspended in SDS-PAGE sample buffer at reducing conditions and electrophoretic analysis was conducted following standard procedures. After SDS-PAGE, gels were either stained with Coomassie blue or transferred electrophoretically to nitrocellulose membranes (Towbin, 1979). Membranes were incubated with anti-AGP MAb (IgG) (Sigma-Aldrich, St Louis, MO) at a dilution of 1:5.000 and KM93 (dil 1:1000) followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse-HRP; Sigma, St Louis, MO) at a dilution of 1:1000. The protein-antibody complexes were visualized using diaminobenzidine as substrate.

Immunohistochemical analysis

Tumor tissue pieces were fixed in methacarn (methanol, chloroform, acetic acid, 6:3:1, for 2hs), embedded in paraffin and cut into 5 mm serial sections. Deparaffinized sections were treated with 10 mM sodium citrate buffer at 100 °C for 5 minutes, then, they were incubated overnight at 4 °C with anti-AGP monoclonal antibody (mouse; Sigma-Aldrich, St Louis, MO, USA) and KM93 MAb. Controls were incubated with PBS instead of monoclonal antibodies. The whole area of each sample was observed by sequentially examining low power (x10) optical fields and with higher magnifications (x40, x63, x100); the staining of cytoplasm, plasma and nuclear membranes were also evaluated. Cells were considered as positive when at least one of these components was stained; the heterogeneous reactivity was graded according to the positive reaction, intensity and distribution. Staining intensity was graded as negative, low, moderate and strong.

AGP detection

Serum AGP was measured by radial immuno-diffusion using DIFFU-PLATE plates (Biocientífica, Argentina). AGP values in a range from 43 to 130 mg/dl were considered as normal.

Results

Isolation and purification of AGP from tumor samples

Electrophoretic separation followed by Western blot analysis and incubation with anti-AGP MAb was performed at the different steps of isolation and purification procedures. In tumor homogenates, subcellular fractions, ammonium sulphate supernatants as well as in glycine-HCl fraction eluted from the Protein A-Sepharose CL-4B, AGP was detected as a band at an approximate molecular weight (MW) of 45kDa (Fig. 2). When incubated with anti-sLex MAb (KM93), these fractions showed a band of reaction at the same MW, although this signal was lower than the one obtained with anti-AGP MAb (Fig. 2).

Immunohistochemical results

Simultaneously to the isolation and purification procedures, a comparative immunohistochemical analysis was performed in colorectal, adenoma and

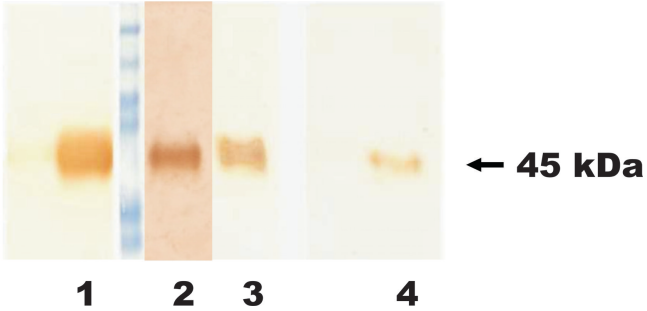

Fig. 2. SDS-PAGE and Western-blotting of fractions obtained at different stages of AGP isolation and purification. **1:** Homogenate of an adenocarcinoma sample incubated with anti-AGP MAb; **2:** AGP-anti-AGP immune complexes eluted with glycine-HCl buffer fraction incubated with anti-AGP MAb; and **3:** AGP-anti-AGP immune complexes eluted with glycine-HCl buffer fraction incubated with anti-sLe x MAb (KM93). A positive reaction at approximately 45kDa is observed.

Table 1. AGP and sLex expression in malignant, benign and normal colorectal tissue samples.

DIAGNOSIS	TISSUE AGP/sLewis x EXPRESSION			
	AGP+/sLex+	AGP-/sLex+	AGP+/sLex-	AGP-/sLex-
Carcinoma	17	10	45	16
Adenoma	7	2	4	9
Normal	1	0	7	15

normal colonic mucosa paraffinized samples. Results are summarized in Table 1. A multivariate analysis employing PCA with Kendall correlations was developed and a statistically significant positive correlation between AGP and sLex tissue expression was found in malignant, benign and normal samples (τ=0.4278).

AGP was detected in 8/23 (35%) normal specimens; expression was restricted to some gland cells and focal in the cytoplasm; frequently, the nuclear membrane was also stained (Fig. 3A). Most cells showed an apical reaction although in some cases, a basal and perinuclear reaction was found; the intensity of the staining was mainly low.

Only one normal sample reacted with anti-sLewis x MAb (1/23, 4%); a focal expression was found localized to the apical part (membrane as well as cytoplasm).

Colorectal adenomas

Eleven out of 22 colorectal adenoma samples reacted positively with anti-AGP MAb (50%) (Fig. 3B); specimens showed staining restricted to some cells with a variable intensity, mainly low; in most cases, reactivity was detected at the basal cytoplasm and perinuclear level of absorptive cells; sometimes, reaction was also shown at the apical surface as well as in the secretion. In a few samples, mucosa cells were also reactive showing the same pattern.

SLex expression was only found in some cells of a very few areas of nine benign specimens (41%); reactivity was mainly restricted to the apical surface; intensity varied from low to moderate.

Carcinoma

Sixty two out of 88 carcinoma samples showed AGP expression (62/88, 70%); in relation to TNM staging, 48% stage II patients showed positive results, 69% stage III patients while 67% stage IV expressed AGP.

Reactive specimens showed a staining comprising most of the cells with a homogeneous cytoplasmic reaction in basal, lateral and apical parts; in some samples, perinuclear and plasma membranes were also reactive; in most samples a strong reaction (Fig. 3C) was found while a few specimens showed a moderate intensity.

On the other hand, 27 sLex positive specimens were detected (27/88, 31%); when positive results were considered according with TNM staging, 31% stage II patients showed positive results, 44% stage III patients while 67% stage IV expressed sLex. Reaction was restricted to several plasmatic membranes in some areas (Fig. 3D).

In three group of samples (carcinoma, adenoma and normal) included, between AGP and sLex expression, a significant correlation through Statistical Multivariate Analysis was found, τ=0.4728.

AGP serum levels

In a group of serum samples AGP was measured and

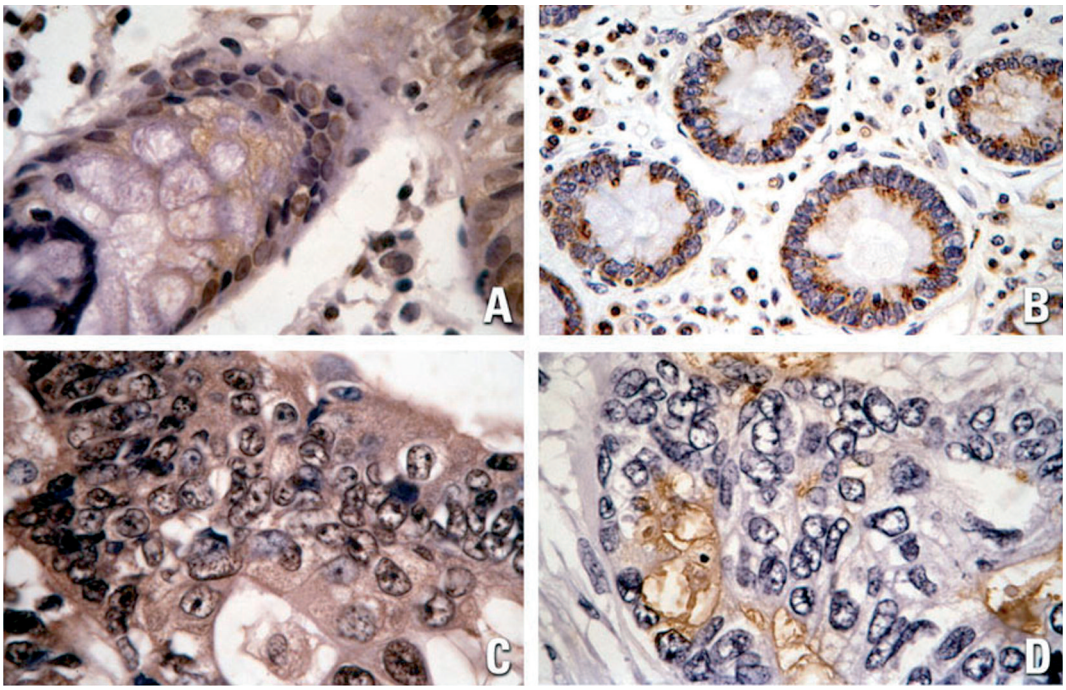

Fig. 3. A. Normal colonic tissue section incubated with anti-AGP MAb. A positive reaction is observed at the cytoplasm and perinuclear part. x 100. **B.** A positive area of an adenoma incubated with anti-AGP MAb which shows a cytoplasmic staining mainly at basal and supranuclear part. In the estroma, a chronic inflammatory infiltrate is depicted which shows AGP positive cells. x 40. **C.** Colorectal adenocarcinoma tissue section reactive with anti-AGP MAb. A strong homogeneous positive staining is observed comprising the whole cytoplasm; some plasma and perinuclear membranes are also reactive. x 100. **D.** Section of a colorectal adenocarcinoma incubated with anti-sialyl Lewis x MAb (KM93). An intense positive reaction mainly at apical plasma membranes and cellular debris is observed; some cells show a strong reaction at basal and perinuclear cytoplasm. x 100

8 out of 35 (23%) patients with colorectal cancer presented abnormal values, one patient had AGP values below normal (19 mg/dl) and two near the lower limit (42 mg/dl) while five showed increased levels.

Patients with benign tumors had normal values with the exception of one case which presented a value near the upper limit (148 mg/dl) while samples belonging to normal individuals showed levels between 43 and 130 mg/dl although one sample was near the lower limit (26 mg/dl) while the other was increased (165 mg/dl).

Statistical comparative analysis between positive tissue expression and increased serum levels did not show a correlation between both parameters (τ =0,0693).

Discussion

In the present research, we report results according with the hypothesis that AGP may constitute a carrier of sLex in colorectal cancer. We show the isolation and purification of AGP from colorectal carcinoma tumor samples having an approximate molecular weight of 45 kDa which is also the site for the binding of an anti-sLex MAb (KM93). Simultaneously, a statistically significant correlation between AGP and sLex tissue expression was found; furthermore, following the well-established model of cancer development in the colon and rectum, the so-called adenoma-carcinoma sequence (Muto et al., 1975), immunohistochemical results showed an increase in AGP through normal mucosa to benign samples and malignant tissues which reached the highest levels. Respect to sLex expression, the number of positive cells as well as the intensity of the reaction in adenoma specimens were much lower than in malignant specimens. Furthermore, in carcinoma patients, we have found an increased number of sLex positive samples from stage II patients to stage III reaching the highest staining in stage IV patients.

With the purpose of study sLex expression in the adenoma-carcinoma sequence many studies have been performed. Yuan et al. (1987) found a frequent expression of sLex and Lex antigens in adenomatous polyps which are considered as premalignant; furthermore, staining frequencies were significantly correlated with increasing polyp size and, in most cases, with increasing severity of dysplasia. Other reports (Hanski et al., 1990; Hanisch et al., 1992) have established a correlation of an anti-sLex MAb (AM-3) reactivity with the progression of dysplasia in adenomatous polyps; they also argued that this MAb can be regarded as a marker for early detection of colonic carcinogenesis. Furthermore, using AM-3 MAb, Baldus et al. (1995) found that adenomatous polyps with the highest risk of malignant transformation strongly expressed sLex (86% of positive specimens) while carcinoma samples reached 96% of positivity. In the present report, we found lower levels either in adenomas (41%) as well as in carcinomas (31%) employing KM93 MAb. In this sense, Baldus et al. (2002) reported contradictory results to the previous study (Baldus et al., 1995) since they did not find a significant correlation between sLex and the development of high-grade dysplasia in colon adenoma employing another anti-sLex mAb (CD15s).

Moreover, with regard to the role of sLex as a marker of tumor progression and prognosis of colorectal carcinoma, Nakamori et al. (1993) reported that an increased expression of this antigen correlated with poor survival; with FH6 MAb they detected 50/132 (37.9%) positive colorectal carcinoma specimens which belonged to patients which a 5-year survival rate of 48.9%, whereas the rate was 86.2% for the patients of the sLex-negative group. In a further study, Nakamori et al. (1997) detected of 159 colon carcinoma samples, 58 (36.5%) were sLex positive and 101 (63.5%) were sLex negative; positive tumors were significantly more likely to be undifferentiated, to have an invasive mode in depth, to metastasize to lymph nodes, to present lymphatic and venous invasion while five year disease-free survival rate was 55.6% compared to 89% in sLex negative patients; they found that sLex might serve as a predictive factor for recurrence. In coincidence, Nakagoe et al. (2000) reported that the survival time after surgery of patients with sLex-expressing tumors was significantly shorter than the survival time of patients with sLex-negative tumors. Baldus et al. (2002) found that in colorectal adenocarcinoma, expression of sLex and sLea antigens was associated with a lower survival probability, especially in patients exhibiting more than 35% of the tumor area although it was not statistically significant.

Colon carbohydrates are heterogeneous and consist of both core and peripheral antigens which suffer alterations in their expression during carcinogenesis and tumor progression. Peripheral antigens include sLea (siaα3Galβ3[Fucα4]GlcNAc) and sLex (siaα3Gaα4 [Fuc4]GlcNAc) and they are expressed on different glycoproteins and glycolipids. MUC1 and MUC2 have been shown to be sLex positive mucins in colon carcinoma (Baekstrom et al., 1991; Hanski et al., 1993, 1995; Baldus et al., 2002) while AGP was also described as another carrier of sLex (Hanski et al. 1995).

Bresalier et al. (1996) demonstrated that sialylated mucin-associated carbohydrate structures are characteristic of colon cancer metastasis and that these results from the selective metastasis of colon cancer cells that express these antigens. They suggested that sialylated carbohydrate structures on mucin play a role in adhesive interactions involving both basement membrane and endothelial-associated ligands which may contribute to colorectal cancer metastasis.

In a previous research (Croce et al., 2001), we detected AGP from colorectal and head and neck carcinoma extranuclear membranes; after incubation with polyclonal anti-AGP antibody, a smear positive reaction at less than 45kD was detected with a main band at approximately the same molecular weight.

AGP shows abnormal levels in cancer patients including colorectal localization (Stamatidis et al., 1990, Stanciu et al., 1990) although values are not so high as those detected in infections and autoimmune disorders;

AGP has been also detected in association with specific antibodies in serum samples belonging to patients with advanced esophagus cancer (Croce and Segal-Eiras, 1997).

In the present research, by immunohistochemical study, we demonstrated that AGP expression comprised most cells with a homogeneous cytoplasmic and membrane reaction and in most cases, a perinuclear staining was also found. This reaction could be related with the hypothesis that malignant AGP may be produced by cancer cells; furthermore, the relatively low AGP serum levels as well as the finding of no statistical correlation between tissue and serum samples may be consistent with the possible role of AGP in dissemination of colorectal cancer. In concordance with this, we have already found (Croce et al., 2001) that metastatic cells present in lymph nodes of larynx and colorectal cancer expressed AGP.

AGP, an acute phase protein, can be induced by any change in homeostasis; human hepatocytes are normally the source of serum AGP and it is known that the gene encoding this protein is positively controlled at transcriptional level by IL-1, IL-6, TNF alpha and hormones; the role of AGP may be related to control the inflammatory process by a feedback inhibitory effect (Walz et al., 1990). It has been described that malignant cells (Ljunberg et al., 1998, Woods et al. 1998) may synthesize different interleukins including IL-6 as well as AGP (Chandrasekaran et al., 1984). Previous reports (Van Dijk et al., 1991) demonstrated that changes in glycosylation of acute phase glycoproteins result from cytokine-induced variations in their biosynthesis in the liver and moreover, De Graaf et al. (1993) demonstrated that inflammation induced an increase in sLex-substituted glycans on AGP.

Development and progression of a malignant tumor constitutes a completely different event respect to inflammatory diseases although it is known that a chronic reaction is usually present in early tumors. Among other factors, cancer cells survive and progress due to their capability to synthesize a complex interleukin network as well as to express molecules related to the metastatic phenotype. AGP may constitute the result of cancer cells interleukin production such as IL-1, IL-6, TNF alpha and, through the induced expression of sLex, AGP may contribute to cancer dissemination.

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