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Immunocytochemical Study of Transforming Growth Factor Expression in Benign and Malignant Gliomas

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Immunocytochemical studies using polyclonal antibodies to epidermal growth factor (EGF) and transforming growth factor (TGF) alpha and beta were performed on 20 cases of human gliomas. EGF immunoreactive material was detected in both benign and malignant glial tumors. In addition, EGF immunoreactive material was detected in normal brain. TGF-beta was detected in both benign and malignant tumors, but was not detected in normal brain. In contrast, TGF-alpha was highly conserved in its expression, occurring predominantly in malignant compared with benign or normal brain tissue ($P < 0.0001$). In malignant gliomas, glioblastomas contained 76% TGF-alpha reactivity (immunoreactive product), and anaplastic types contained 85% reactivity. Benign gliomas contained only 13% TGF-alpha reactivity. These findings support the role of TGF-alpha as an oncoprotein marker in brain neoplasms. (Am J Pathol 1989, 134:895–902)

Transforming growth factors (TGF) are low molecular weight polypeptides that reversibly induce anchorage-independent growth of nontransformed anchorage-dependent cells. At least two types of transforming growth factors have been described—alpha and beta. TGF alpha is a M_r 6000 single-chain polypeptide that shares 30 to 35% homology with human and rat epidermal growth factor (EGF).[1] Both EGF and TGF-alpha can compete for the same membrane receptors.[2] In contrast to EGF, TGF-alpha secretion is seen primarily in the transformation of cells to a malignant phenotype. The release of TGF-alpha by transformed cells is thought to be the result of oncogene activation rather than a consequence of a cellular change during the process of transformation.[3] The link between oncogenes and growth factors has been established for many polypeptide growth factors.[4] The autonomous production of growth factors has led to the autocrine mechanism for neoplastic conversion.

To obtain a more thorough understanding of the ubiquitous patterns of polypeptide growth factor production, we performed an immunocytochemical analysis in 20 cases of benign and malignant human glial tumors. Glial tumors were chosen as a model of study because of their precise morphologic correlation relating to malignant phenotypes.[5]

Materials and Methods

Twenty human glioma biopsies obtained from archival cases within the Department of Pathology at the Medical College of Georgia from 1981 to 1984 were examined and classified by a neuropathologist. Of the 20 specimens, 8 were grade I (benign), 5 were grade II (anaplastic), and 7 were grade III (glioblastoma multiforme). This grading system corresponds to that system recommended by the World Health Organization.[6] Anti-TGF-alpha, beta, and EGF polyclonal antibodies were obtained from Biotope Company (Bellevue, WA). These antibodies were generated from synthetically produced polypeptides. The sensitivity of these polyclonal antibodies was determined by Western blot analysis. The detection limit of these reagents is between 5 and 10 ng of antigen. EGF does not react with this antisera when examined similarly.[7] For these studies, we used an indirect peroxidase-anti-peroxidase method described previously.[8,9] Briefly, sections are deparafinized in 100% xylene, and then rehydrated by graded series of ethanols (100 to 50%). The slides containing 5-μ thick sections are then placed in a 0.05 M TRIS, 1.5% saline, pH 7.6 buffer before the start of the procedure. The sections are then blocked by the addition of 5% powdered milk dissolved in TRIS-saline for 30 minutes. This is followed by the addition of 0.3% H_2O_2 for 30 min-

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utes to block endogenous peroxidase. After several rinses in TRIS-saline, the slides are reacted with the antibody (1:10 dilution in TRIS-saline). Control sections were reacted with goat serum. The antibodies are visualized by sandwiching with either anti-goat or anti-rabbit linking agents. The labeling agent, peroxidase to anti-peroxidase immunoenzyme complex, is then added. The complex is then visualized by the addition of a chromogenic substrate (amino-ethyl carbazole, AEC). All sections were then counterstained with hematoxylin and mounted with Permount and then analysed.

Color Image Analysis System

Sections of tumor were examined in three high-power fields, consisting of approximately 100 cells/high power field. Cytoplasmic immunoreactive product was quantitated using a Delta Scan Color Image Analyzer (Delta Technologies, Inc., Augusta, GA). This system includes a color video camera mounted on a Zeiss Axiophot microscope. In turn, the video camera is cabled to the input of an AT&T True Vision Image Capture Board, installed on an IBM-PC with a hard disk and Microsoft mouse. The output of the ICB is directed to a Sony RGB color monitor. To maintain constant light intensity conditions, the recorded image was performed in one sitting. Only the focus was changed from specimen to specimen to insure that all samples were recorded on the exact magnification and illumination intensity. Each digitized image was then immediately saved to hard disk for additional analysis. Verification of the accuracy of the reaction product was always confirmed by pixel mapping of the localized reaction product.

Color Image Analysis Software

Several investigative methods have been used to quantitate immunologic staining in tissue sections.[9,10] To obtain objective quantitation, image processing techniques that use monochrome optical density for measuring intensity have been used.[11,12] This approach can analyze both real-time and stored images. Pixel measurements are generated within an object by high resolution digital sampling. We have developed a video-based microscope system, driven by color algorithms structured in C language. This software system can accurately quantitate by pixel mapping digitized images received from the videomicroscope hardware. This system can analyze both real-time and stored images. Pixel measurements are generated within an object by high resolution digital sampling.

The software used for this analysis was developed by one author (SB) and is called "CoreSCAN." CoreSCAN is a color video analysis program. The program compares each scanned pixel to a previously collected list of color values. If the color value of the pixel matches the value within the list, the program increments a net pixel count. The net pixel count may be output as a numerical value in operator-specified units. Each matching pixel also is included in a map which may be displayed as an image overlay. The operator creates a list of color values for comparison in a controlled collection process. The color-banding feature of CoreSCAN may be used to augment the collection and comparison of color values. Color banding reduces the potential number of unique colors contained in an image, from the theoretical maximum of 32,768 to either 4096, 512, 64, or 8 colors. This effectively coerces similar color values to identical color values, thereby reducing the size of the list of color values used in comparison and decreasing the comparison time.

Statistical Analysis

An analysis of the F ratios of variance was used to determine the acceptable selection of the sample size. Descriptive statistics were calculated to determine the mean variance of standard deviation and standard error of the means. Statistical differences between the means were determined by a multiple analysis of variance (MANOVA, followed by Tukey multiple range tests, using StatGraphics [SBSC and Company]). Means deemed significant were greater than $P < 0.05$.

Results

Demonstration of quantitated immunoreactive product by color video analysis is illustrated in Figures 1 to 4. Figure 1 is a digitized image of an anaplastic (grade II) astrocytoma stained with goat anti-TGF-alpha, demonstrating moderate localization of immunoreactive product. Once the specific colors of interest are placed within the color net, CoreSCAN will digitize the image and color-pixel map the area of reaction product (Figure 2). Figure 3 shows the digitized image of a malignant (grade III) astrocytoma stained with goat anti-TGF-alpha, with extensive amounts of immunoreactive product. Computer analysis of the same image of Figure 3 reveals the TGF reaction product that has been localized and quantitated and indicates the specificity of the color video analysis system (Figure 4).

The results of quantitative color video analysis of EGF, TGF-alpha and beta, are illustrated in Figures 5 to 8. EGF staining patterns were examined in both normal and neo-

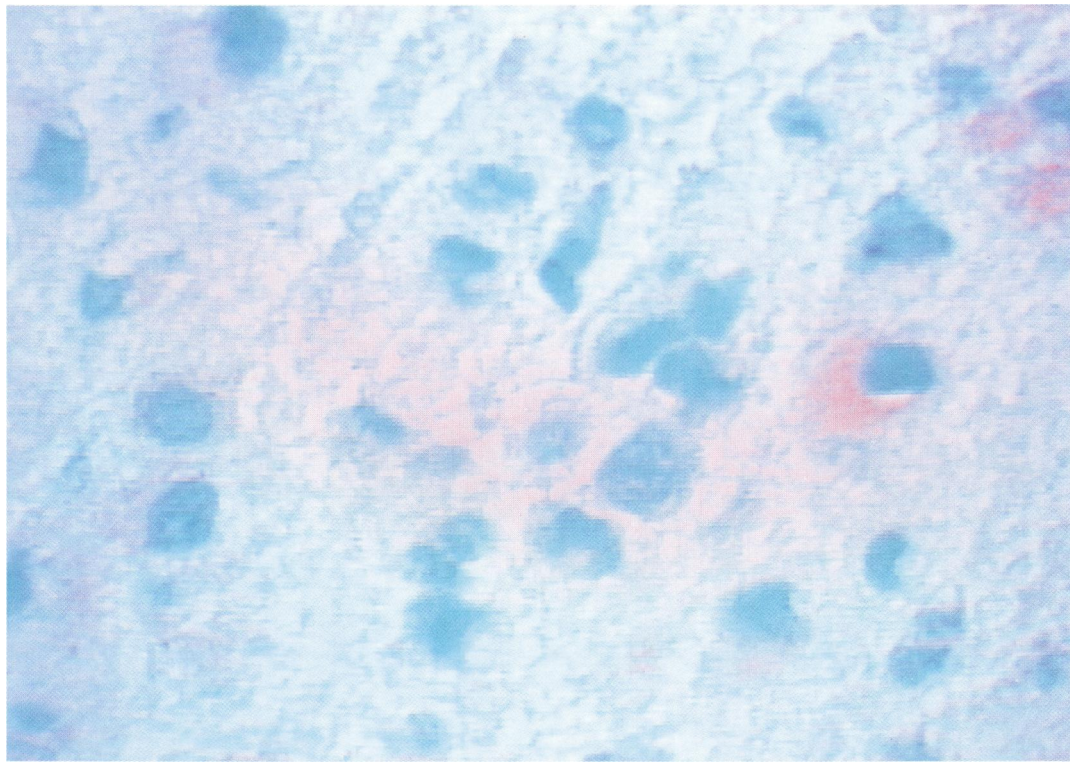

Figure 1. *Digitized image of anti-TGF alpha staining of an anaplastic astrocytoma (counterstained with hematoxylin, ×400).*

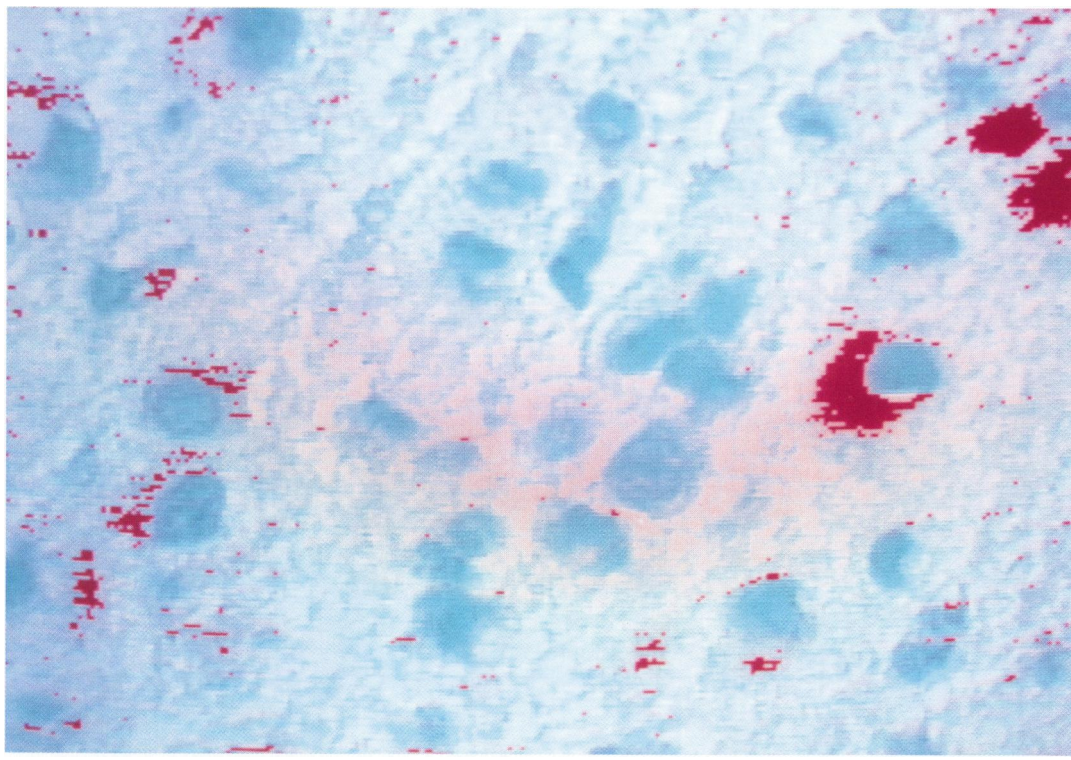

Figure 2. *Color pixel mapping of immunocytochemical reaction product of the same image in Figure 1.*

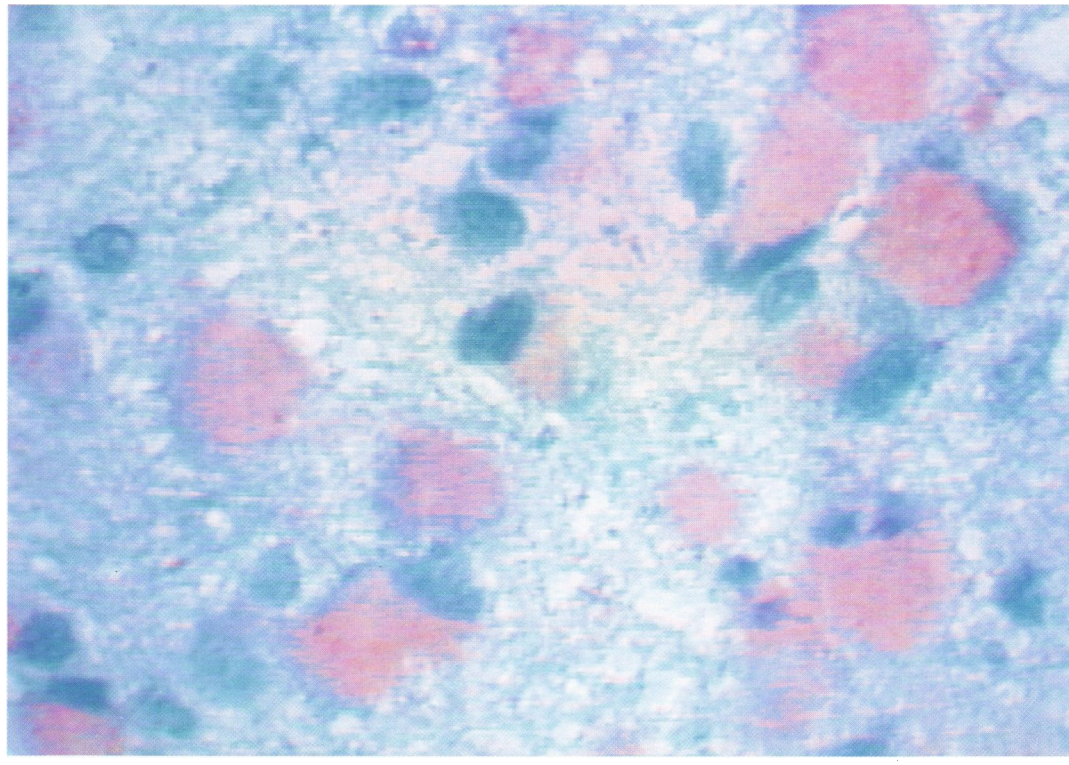

Figure 3. *Digitized image of anti-TGF-alpha staining of a malignant astrocytoma, grade III (×400).*

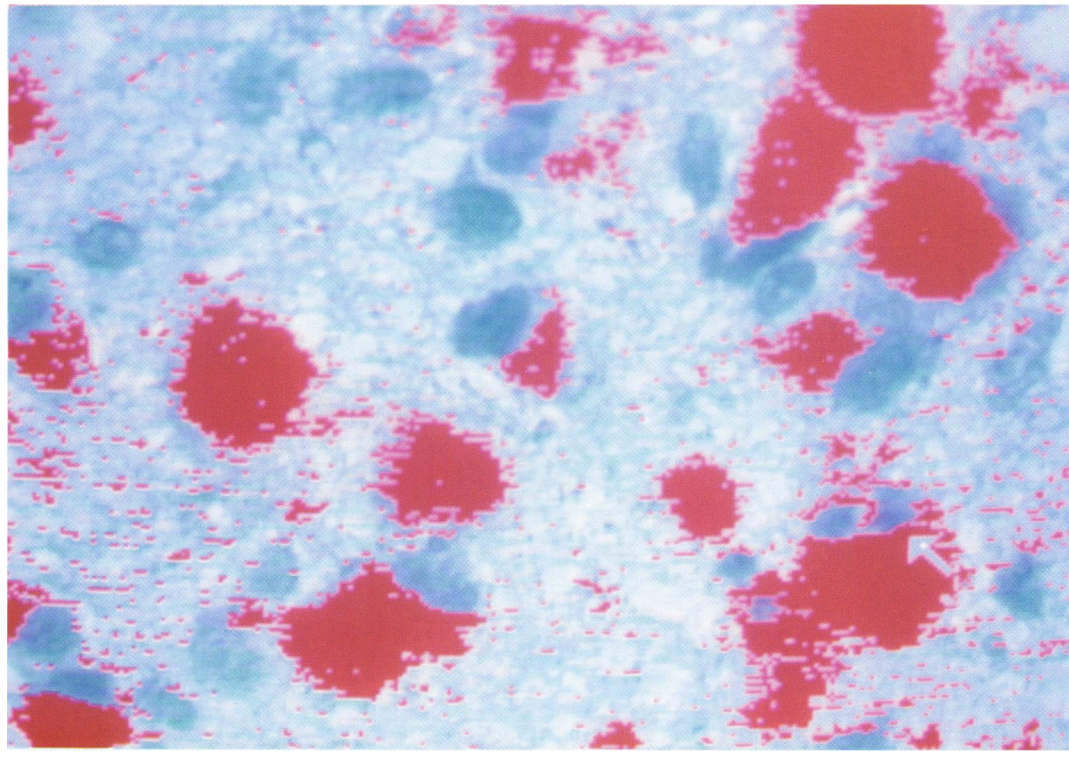

Figure 4. *Computer analysis with resultant pixel mapping of the same image as in Figure 3.*

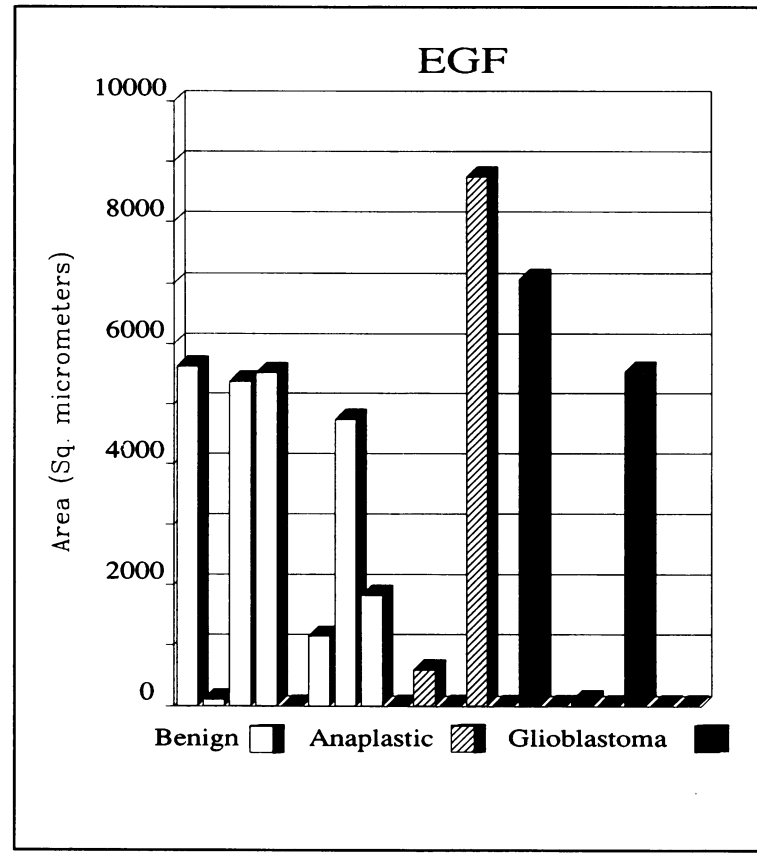

Figure 5. *Distribution EGF staining patterns of both benign and malignant gliomas. Results are given as the mean ± standard error of the mean (SEM) of three observations per group.*

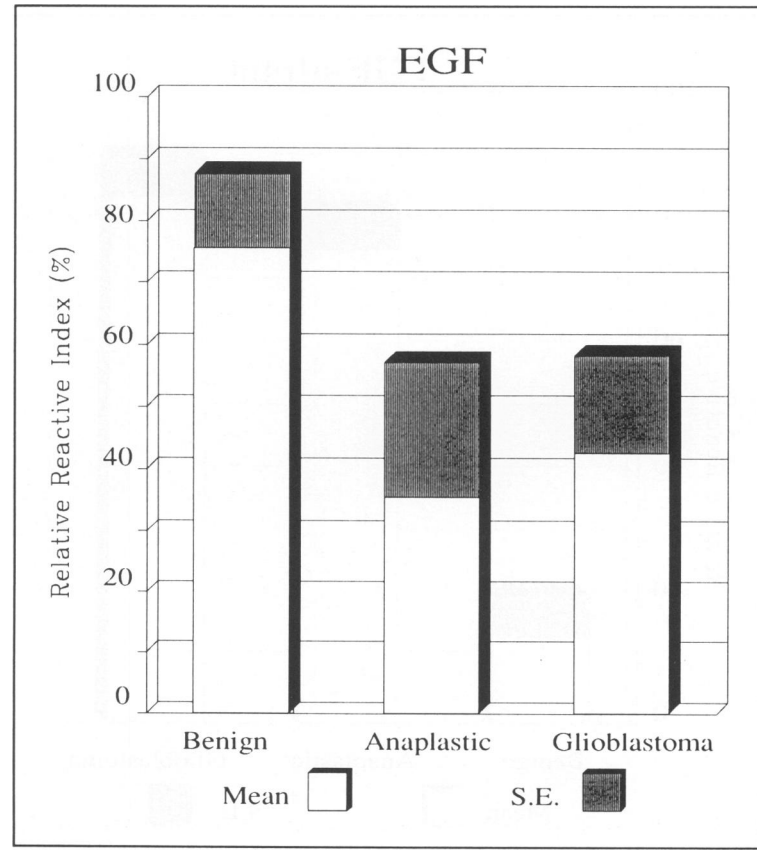

Figure 6. *Relative levels of EGF immunoreactive product in all grades of astrocytomas. Statistical differences between groups were determined by MANOVA and Tukey multiple range tests.*

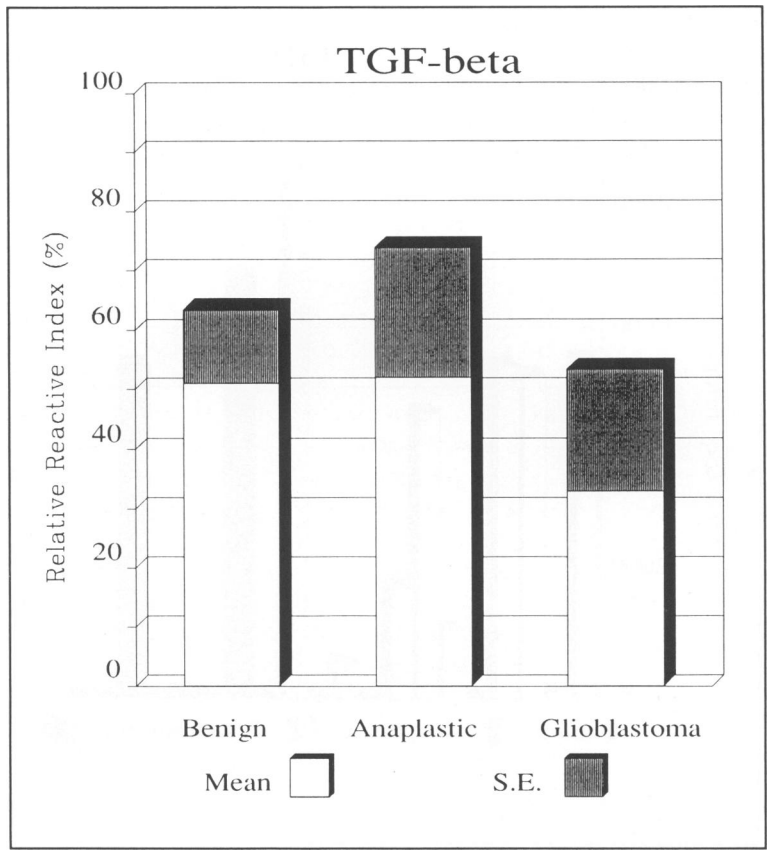

Figure 7. *Quantitative distribution of TGF-beta staining patterns of both benign and malignant gliomas.*

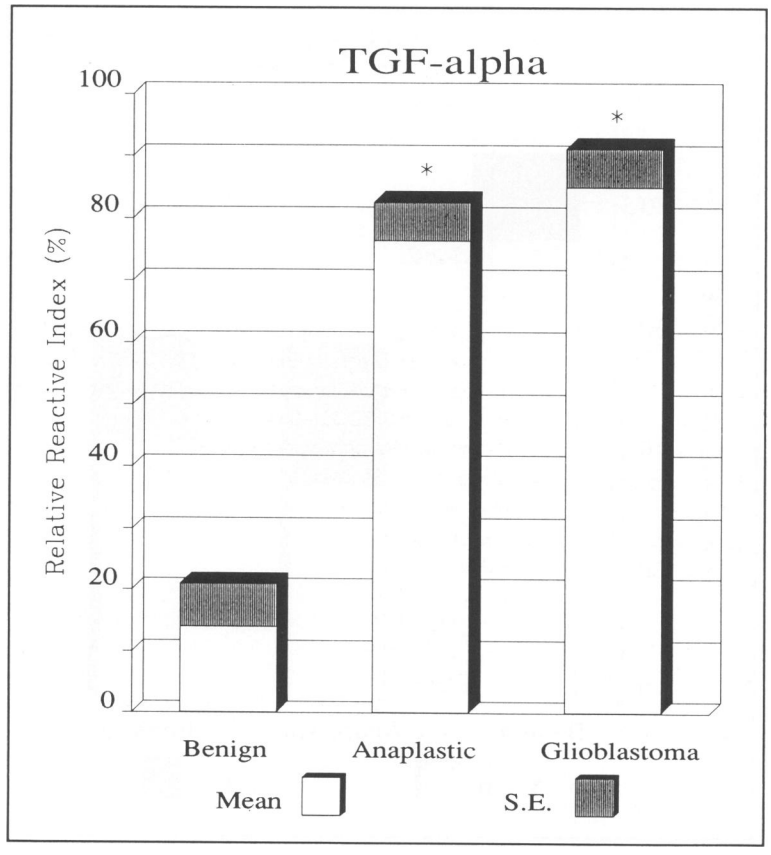

Figure 8. *Relative levels of TGF-alpha immunoreactive product in all grades of astrocytomas.*

plastic brain tissue first. EGF immunoreactive sites were found in the cerebrum and cerebellum of normal human brain tissue. White matter tended to contain greater amounts of reactive material. Control sections reacted with goat serum or TRIS buffer alone did not stain. Figure 5 depicts the area (in square micrometers) of EGF-reactive material detected in 20 cases of benign and malignant gliomas. These cases are grouped according to grades of malignancy. Each bar is a mean value of a triplicate measurement by color video analysis. Immunocytochemical reactive material for EGF was detected in all human gliomas. However, the benign tumors produced far more EGF reaction product than malignant tumors (mean area of reactivity for benign tumors equaled 17,652 sq μ per area of scanned field *vs.* 8493 sq μ per area of scanned field for malignant tumors). These benign to malignant categories produced a trend of decreasing EGF reactivity. When these values were normalized by comparing them with the most reactive tumor within the group, these differences were not statistically significant (Figure 6). Benign tumors had 75.6% relative reactivity, whereas malignant tumors had 35.4% and 42.6%, respectively.

We next investigated the cellular localization of TGF-alpha and TGF-beta on the same glial tumors. TGF-beta immunoreactive material was detected in all grades of glioma without any demonstrable correlation to the degree of malignancy (Figure 7). These findings confirmed the findings of previous studies by other investigators,[13] and also extend the localization to benign tumors as well. In addition, TGF-beta tended to stain mesenchymal structures such as fibroblasts and vascular cells within the brain tumors.

In contrast, immunocytochemical localization of TGF-alpha to the same groups of tumors revealed a tumor-specific distribution. Immunoreactive material was always localized in cells of malignant gliomas. Benign gliomas were consistently negative. The mean area of reactivity was quantified by triplicate analysis using color video analysis. The mean values were 1651 sq μ per scanned area for malignant gliomas (anaplastic and glioblastoma), and 11.1 sq μ per scanned area for benign gliomas. The individual absolute values were again normalized by comparing to the most immunoreactive section. These TGF-alpha immunocytochemical values were then examined statistically (Figure 8), and were shown to be significantly different ($P < 0.0001$). TGF-alpha immunoreactive product easily segregated benign gliomas from both malignant gliomas. Both malignant gliomas (anaplastic and glioblastoma) demonstrated a relative reactivity index of 76 and 85%, whereas benign gliomas showed only 13% reactivity.

Discussion

These findings support the role of a functional classification of tumors based on the quantitated patterns of polypeptide growth factor localization. Furthermore, they lend support to the oncogene concept of neoplastic transformation and progression to a more malignant phenotype type that occurs via gene amplification and translocation of growth factor signals.[13,14] The separation of benign from malignant gliomas using TGF-alpha production is further supported by current known biology and physiology of this polypeptide.[15] Recently, Lieberman and coworkers[15] have shown that the majority of malignant glioma cell lines express high levels of a 4.6 kb TGF-alpha-specific mRNA. TGF-alpha mRNA was not present in normal or benign tumors. Other investigators have implicated TGF-alpha an angiogenic growth factor.[17,18] Massive proliferation of endothelial cells is characteristic of glial malignancies.[6] Hence, the presence of TGF-alpha and other known growth factors for malignant glial cells may influence tumor progression.

TGF-beta has been identified in malignant gliomas by several investigators. In our study, TGF-beta was present in malignant tumors and benign tumors, but not in normal brain. Our findings are in agreement with those of Clark and Bressler,[20] who found TGF-beta activity in a variety of grades of gliomas.

The use of *in situ* immunocytochemical methods allows precise localization of immunoreactive products in question. The results reported herein supports the use of TGFs localization as a chemical means of separating benign from malignant glial tumors.

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