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EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR AND C-ERBB-2 ONCOPROTEIN IN HUMAN TUMORS.

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The EGF-binding capacity and the phosphokinase activity associated with the EGF receptor (EGF-R) have been evaluated in human tumors of various origins.

1) Tumors of the Central Nervous System.

In glioblastomas and high grade astrocytomas, the mean value of EGF binding capacity was higher than in low grade astrocytomas. Significant amounts of EGF-R were also found in meningiomas. In the tumors of glial origin, a positive correlation also exists between the autophosphorylation of the receptor and the degree of malignancy.

2) Breast cancer.

By EGF-binding assay, EGF-R is found in approximately 35% of breast tumors. In the present study, about 90% of the tumor samples (122/134) exhibited a significant activity of the EGF-R associated kinase activity. The kinase activity was not statistically different in tumor and in adjacent "normal" tissue. There was a negative relation between the expression of EGF-R and the amount of steroid hormone receptors.

c-erbB-2 oncoprotein has been detected by Western blot in a series of tumor samples and in some cases, to a lesser extent in adjacent breast tissue. Our results suggest an association of c-erbB-2 with the process of transformation in human breast tumors.

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EPIDERMAL GROWTH FACTOR RECEPTOR ASSAY : VALIDATION OF A SINGLE POINT SATURATION METHOD IN BREAST CANCER

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In breast cancer, the presence of epidermal growth factor receptor (EGFR) has been demonstrated to be associated with a poor prognosis. Its determination brings an additional prognostic value to that of steroid receptors. Usually EGFR are measured by competition with labeled EGF according to the multipoint Scatchard Analysis (SA). This method limits EGFR investigations because of the substantial quantity of tissue required (500-1000 mg). We thus adapted and validated a single saturating dose technique (SSDT) suitable for large-scale clinical studies. EGF binding was analysed using human recombinant EGF, in 120 human primary breast cancers, of which 41 were checked with both SSDT and SA. For routine analysis, only 100-150 mg of tissue were required. Optimal conditions were as follows : 100 µl of labeled EGF (1nM) incubated with 100 µl of the membrane preparation in absence or in presence of an excess of unlabeled EGF (160 nM), 1.5 hrs at 22°C.

EGFR assay by SSDT showed a unimodal distribution, with a progressive decrease in high values (range = 0-1448, median = 10, fmol/mg membrane proteins). Coefficients of variation for the intra-assay reproducibility varied from 5.14 to 17.78 %. SA performed on 56 tumors exhibited 79 % of tumors expressing a single high-affinity site (mean Kd = 0.6 nM) and 21 % expressing an additional low-affinity site (mean Kd = 3.3 nM). SSDT optimisation for labeled EGF concentration showed that 1 nM was the best compromise to saturate the high-affinity sites without interference with the low ones. There was a good quantitative correlation between SSDT and SA ($r = 0.973$, $p < 0.001$) in spite of an underestimation of EGFR by SSDT relative to SA. On the whole population we confirmed a significant inverse correlation between EGFR and estradiol receptor levels (Kendall rank correlation, $p < 0.001$). This simplified method, requiring small quantities of tissue, could allow the clinical interest of EGFR determination to be investigated on larger populations.

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HYDROXYLAPATITE ASSAY TO MEASURE EPIDERMAL GROWTH FACTOR RECEPTORS IN MEMBRANE PREPARATIONS

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We have introduced a method in which Hydroxylapatite (HAP) is used for separation of Epidermal Growth Factor Receptor (EGFR) bound ^{125}I-EGF from non-bound ^{125}I-EGF. Leaving the non-bound ligand in solution, HAP adsorps the EGFR protein containing membranes. Only low-speed centrifugation is subsequently required for the separation step, whereas other methods use high-speed centrifugation for the separation of bound and free ligand. Experimental conditions, involving bacitracin-containing buffer to minimize binding of ^{125}I-EGF to the HAP, have been established for the determination of EGFR in human placental and breast tumor membrane preparations. Also, the HAP-assay was used in self-displacement analysis for the determination of the specific activity and maximum binding ability of Protag-125 iodinated EGF, respectively 77.2 ± 16.8 µCi/µg and 96 ± 2 % (n=10).

One-hundred µl aliquots of the membrane preparations, after addition of BSA to a final concentration of 0.1% were incubated in a final volume of 140 µl for 20 hours at 20 °C with increasing concentrations of ^{125}I-EGF ranging from 50 to 2000 pM in the absence and presence of a 250-fold excess non-labeled EGF to correct for non-specific binding. For separation of free and receptor-bound ligand after the overnight incubation, 100 µl HAP suspension was added to each assay tube and incubation was performed for one hour at 20 °C. Following this incubation, wherein the tubes were shaken every ten minutes, the HAP with bound EGF Receptor was precipitated by centrifugation for 2 minutes at 1000xg. The HAP pellets were washed twice with bacitracin containing buffer. Finally, the HAP pellets were counted for radioactivity.

In summary, we report a sensitive method for the measurement of EGFR. The procedure is easy applicable to the pellet fraction which remains after standard preparation of cytosols for routine assay of estrogen and progesterone receptors according to EORTC guidelines. The methodology will enable every laboratory which is currently involved in measurement of steroid receptors to introduce an EGFR assay.

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IODINATION OF EPIDERMAL GROWTH FACTOR USING A MILD GLYCOURIL AGENT

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Iodination of Epidermal Growth Factor (EGF) using a mild glycouril agent (Protag-125 iodination reagent) resulted in a heterogeneous ^{125}I-EGF population (P0), which could be separated into three peaks on a C18 reverse phase HPLC column: P1, P2 and P3.

Preparative isolation of the three peaks revealed three ^{125}I-EGF preparations with different binding properties towards human placental membranes containing Epidermal Growth Factor Receptors. Oxidation of P2 with either hydrogen peroxide or a large amount of Protag (compared to the amount used in the iodination reaction) resulted in a change in elution volume of the tracer: the HPLC profile showed a single peak at the same volume of P1 in the original HPLC profile. Moreover, iodination of the P2-fraction with non-radioactive iodine and Protag shifted the peak to the elution volume of P3. These results strongly indicate that the EGF eluted in peak P1 consists of labeled EGF, which has been oxidized in the iodination reaction and that peak P3 contains ^{125}I-EGF which has been strongly iodinated. Peak P2 probably contains slightly iodinated EGF species and simultaneously oxidized and iodinated EGF molecules. Native EGF eluted at the same volume as P2. The use of more Protag in the iodination reaction led to an increase of peak P1 and P2, while the height of peak P3 decreased, probably due to oxidation of slightly (P2) and strongly (P3) iodinated EGF.

The maximum binding ability of all four ^{125}I-EGF preparations (P0, P1, P2 and P3) to the placenta membranes was nearly 100%, as determined with the Hydroxylapatite-assay of Benraad et al. (to be published). The specific activities of the different ^{125}I-EGF preparations, determined with self-displacement analysis were quite different. The unpurified tracer P0 had a specific activity of 77.2 ± 16.8 µCi/µg (n=10). In a typical experiment P1 had a specific activity of approximately 37 µCi/µg, P2 about 28 µCi/µg and P3 about 260 µCi/µg. P2 and P3 were bound with equal affinity to the human placenta membrane preparations as compared to non-iodinated EGF. The affinity of the P1 tracer towards the membrane receptor was smaller. The P1 component present in P0 caused the lower affinity of P0 as compared to the affinity of native EGF. Therefore, it is recommended to iodinate EGF with a quantity of Protag, at which P1 is negligible, so that oxidized products of the iodination reaction do not interfere in binding assays. The resulting ^{125}I-EGF preparation has excellent properties compared to EGF, which has been iodinated with more harsh iodination reagents such as Chloramin-T.