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SHORT NOTE

Inhibition of EGF-Induced Signal Transduction by Microgravity Is Independent of EGF Receptor Redistribution in the Plasma Membrane of Human A431 Cells

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Epidermal growth factor (EGF)-induced c-*fos* and c-*jun* expression is strongly suppressed in microgravity. We investigate here whether this is due to inhibition of processes occurring during the initiation of EGF-induced signal transduction. For this purpose, EGF-induced receptor clustering is used as a marker. The lateral distribution of EGF receptors is directly visualized at an ultrastructural level by the label-fracture method. Quantification of the receptor distributions shows that EGF-induced receptor redistribution is similar under normal and microgravity conditions. This suggests that microgravity influences EGF-induced signal transduction downstream of EGF binding and EGF receptor redistribution, but upstream of early gene expression in human A431 cells.

INTRODUCTION

Growth factors are involved in the regulation of cell proliferation and differentiation [3, 21]. In general, their response is mediated by transmembrane receptors with inducible tyrosine kinase activity. Like other growth factors, epidermal growth factor (EGF) induces receptor redistribution in the plasma membrane, leading to receptor dimerization [11, 32], which is obligatory for activation of the EGF receptor tyrosine kinase [20, 21]. The EGF receptor tyrosine kinase subsequently phosphorylates substrate proteins that mediate the cellular response to EGF [1, 13, 15, 16]. These events participate in triggering signaling pathways that lead to elevated expression of specific genes, including the nuclear proto-oncogenes c-*fos* and c-*jun* [17]. Ultimately, the continuous presence of EGF induces DNA synthesis and cell division in target cells [3, 18].

The activation of human lymphocytes by concanavalin A initiates molecular mechanisms which show great similarity to those triggered by growth factors [2]. Several reports have indicated that activation of human lymphocytes in response to concanavalin A and other mitogens is almost completely inhibited in microgravity [4, 14]. We have assumed that the observed effects of microgravity on lymphocytes originate from gravity-dependent modulations of one or more events in the molecular route responsible for signal perception and transduction. Using EGF-induced signal transduction in A431 cells as a model system, we have demonstrated that microgravity strongly inhibits EGF-induced c-*fos* and c-*jun* expression in A431 cells [7]. Furthermore, partial activation of signaling pathways by agents which bypass the EGF receptor, such as phorbol 12-myristate 13-acetate (PMA: activator of protein kinase C) and forskolin (activator of protein kinase A) has revealed that distinctive signaling pathways are modulated by microgravity, while others are not [8]. In addition, EGF-induced cell rounding was enhanced in simulated microgravity [19].

In this paper we investigate whether the reduction in EGF-induced c-*fos* and c-*jun* expression in microgravity results from an inhibition of processes occurring at the initiation of the EGF-induced signaling cascade. For this purpose, EGF-induced receptor clustering has been monitored at an ultrastructural level.

MATERIALS AND METHODS

Materials. The mAb 2E9 was characterized as described in detail elsewhere [5, 6]. EGF was from Collaborative Research (Waltham, MA); gelatin, Merck (Darmstadt, FRG); paraformaldehyde, BDH Chemicals (Poole, United Kingdom); rabbit anti-mouse polyclonal antibody, Nordic (Tilburg, The Netherlands); fetal calf serum, Integro (Zaandam, The Netherlands); and Dulbecco's modified Eagle's medium, Flow Laboratories (Irvine, Scotland). Protein A–gold complex was prepared as described elsewhere [35] and obtained from Sigma Chemical Co. (St. Louis, MO).

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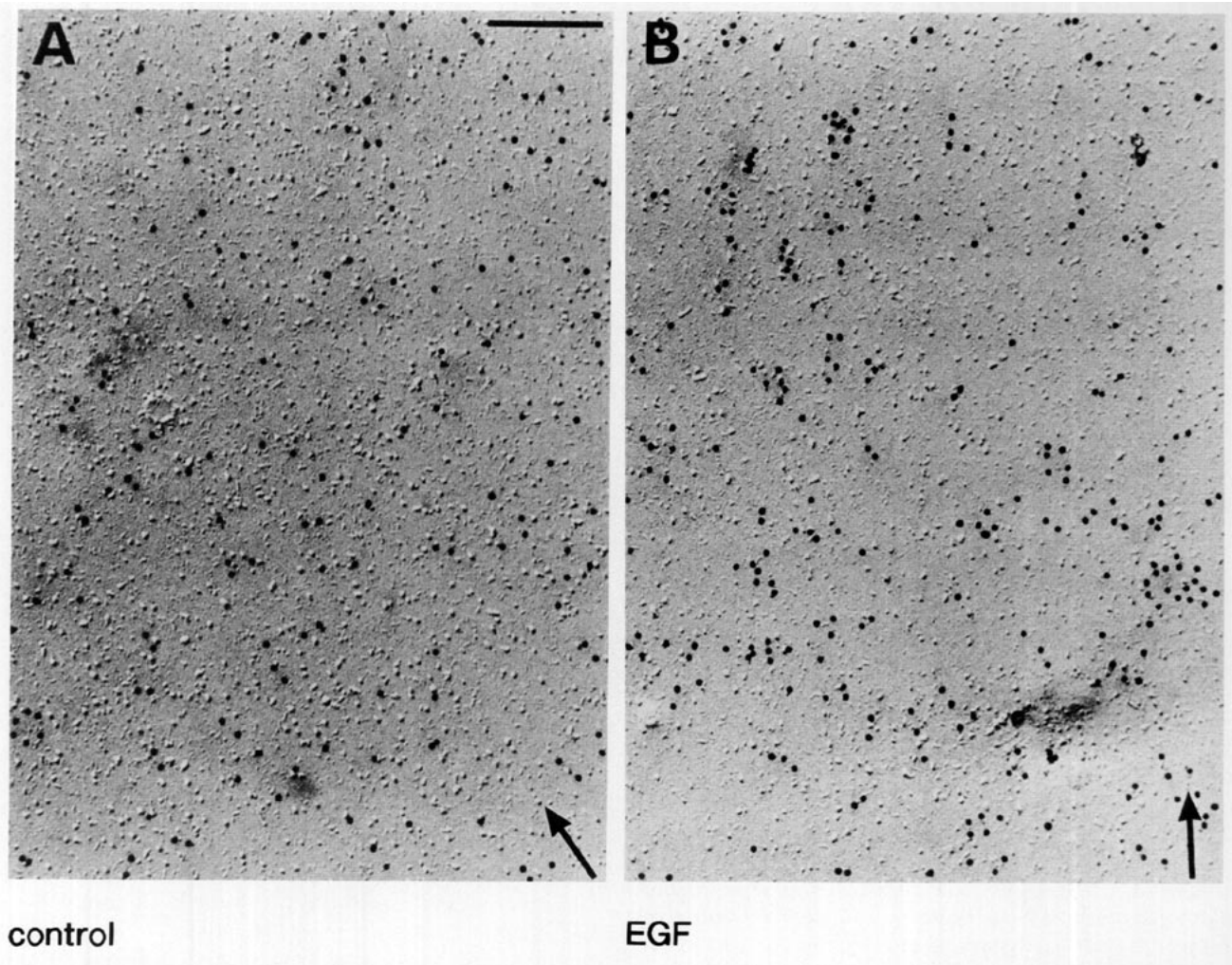

FIG. 1. Rapid EGF-induced receptor clustering in human A431 cells. A431 cells were treated with EGF (80 ng/ml; B) or buffer alone (A) and subsequently fixed, immunogold labeled with mAb 2E9, polyclonal rabbit anti-mouse antibody, and 11-nm protein A colloidal gold, frozen, and freeze-fractured as described [9, 22]. Representative electron micrographs of the exoplasmic fracture face of A431 cells are presented. Bar indicates 200 nm and arrows indicate direction of shadowing.

Cell culture. A431 human epidermoid carcinoma cells (passages 10–40) were grown in DMEM supplemented with 7.5% FCS in a 7% CO_2 humidified atmosphere. Cells were grown for 48 h and cultured until small groups of cells had formed.

Immunogold labeling and freeze fracturing. Freeze-fracture electron microscopy was performed as described previously [17, 32]. Controls to determine specificity of the immunolabeling included cells displaying decreased numbers of EGF receptors, such as normal human fibroblasts. The label density in these cells correlated well with the expected receptor density [22], while immunogold labeling of NIH 3T3 2.2 cells with no detectable endogenous EGF receptor expression was negative. The exoplasmic fracture faces of specimens were photographed in a Philips CM10 electron microscope at 28,500 times magnification.

Quantitative analysis of EGF receptor distribution. Electron microscope images of the exoplasmic fracture face (EFF), representing a membrane area of ~3.6 μm^2, were digitized on-line, by means of the crystal image analysis system on a CM10 electron microscope (Philips, Eindhoven, The Netherlands) and the Z-value of the particle distributions was determined, as described previously [9, 22]. Positive Z-values indicate an aggregated particle distribution, whereas negative Z-values indicate dispersion of gold particles. Absolute Z-values of 1.65, 2.33, and 3.10 indicate 5, 1, and 0.1% significance probabilities, respectively. Further characterization of the lateral distribution of immunogold-labeled EGF receptors was performed by considering particles to be monomers if the distance between their centers was more than 35 nm. Subsequently, the number of single particles (defined as monomers) was counted and expressed as the percentage monomers of the total number of particles (referred to as the percentage monomers). The relation between the percentage monomers and the particle density of the distributions was subsequently determined and differences in particle distributions were assessed by means of covariance analysis.

RESULTS AND DISCUSSION

One of the first cellular responses following binding of EGF to the EGF receptor is a drastic redistribution of cell surface EGF receptors, leading to receptor cluster-

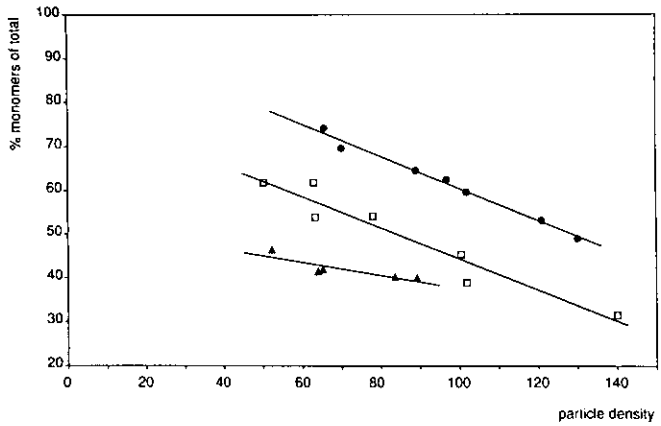

FIG. 2. Quantification of the receptor distribution by determination of the relation between the percentage monomers and the particle density. Random particle distributions of various particle densities were generated by computer. Subsequently, the percentage monomeric particles of the total of a given particle density was determined and plotted as a function of the particle density (closed circles). Each analysis is based on a sample of 12 independent simulations at a given particle density. In addition, A431 cells were treated with buffer or buffer supplemented with EGF (80 ng/ml) for 5 min, fixed, and prepared for quantitative analysis of the lateral EGF receptor distribution. The percentage monomers of 7 and of 5 independent experiments is respectively determined and plotted as a function of the particle density (open squares for control cells; closed triangles for EGF-treated cells). Each data point was based on a sample of 30 to 40 analyses, each performed on a different cell.

ing. Electron micrographs of the extracellular fracture face of A431 cells prepared according to the label-fracture method [17] reveal the lateral distribution of EGF receptors in the plasma membrane of A431 cells, as represented by 11-nm gold particles (Fig. 1A). Within 5 min, EGF (80 ng/ml) induces drastic EGF receptor redistribution, resulting in clustering of receptor-associated gold particles (Fig. 1B).

To quantify the lateral EGF receptor distribution, regression of the percentage of monomeric gold particles of the total number of gold particles on the particle density was determined in control and EGF-treated A431 cells and compared with a random particle distribution. As shown in Fig. 2, the linear regression of the random particle distribution equals $y = -0.37x + 97$, while particle distributions of control and EGF-treated A431 cells equal $y = -0.36x + 80$ and $y = -0.14x + 52$, respectively. Analysis of covariance, using the percentage monomers and the particle density as covariates, shows that these regressions are significantly different, in agreement with the qualitative aspects of EGF-induced receptor redistribution (Fig. 1). Alternatively, the receptor distributions can be characterized by determination of the approximate normal deviate Z [9, 22]. For the random distributions, Z equals -0.1 (SEM = 0.2; Fig. 3). The Z-values for control cells and EGF-treated cells equal 1.3 (SEM = 0.3; Fig. 3) and 3.5 (SEM = 0.5; Fig. 3), respectively, confirming previous findings.

A431 cells were brought into microgravity for 5 min using sounding rocket flight [7]. Samples were fixed either at the beginning of the microgravity phase or after 5 min of treatment with EGF (80 ng/ml) or buffer alone. At the same time, a ground reference experiment was performed under normal gravity conditions. Representative electron micrographs from the EFF of A431 cells in the same experiment show no qualitative differences in plasma membrane topology, intramembranous particle (IMP) distribution, or EGF receptor distribution, as compared to the normal gravity reference samples (Figs. 4A–4F).

The receptor distributions of control and EGF-treated cells under normal gravity conditions were compared to the receptor distributions of cells in microgravity, using the regressions of the particle distributions in control and EGF-treated A431 cells in normal gravity and in microgravity, respectively. The regressions for control and EGF-treated A431 cells in microgravity were $y = -0.36x + 81$ for control cells and $y = -0.17x + 54$ for EGF-treated cells, respectively. To test for a possible effect of microgravity on the EGF receptor distribution of both control (untreated) and EGF-treated samples, analysis of covariance was applied, using two different regression lines for control and EGF-treated cells, respectively. Such analysis demonstrates that microgravity does not significantly affect the receptor dis-

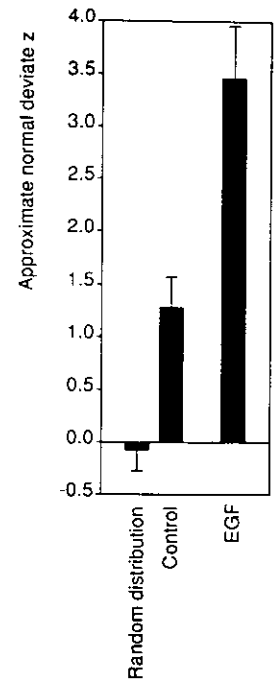

FIG. 3. Quantification of the receptor distribution by determination of the approximate normal deviate Z. The mean approximate normal deviate Z of the random particle distributions and the gold particle distributions of control and EGF-treated cells is determined as described previously [9, 22].

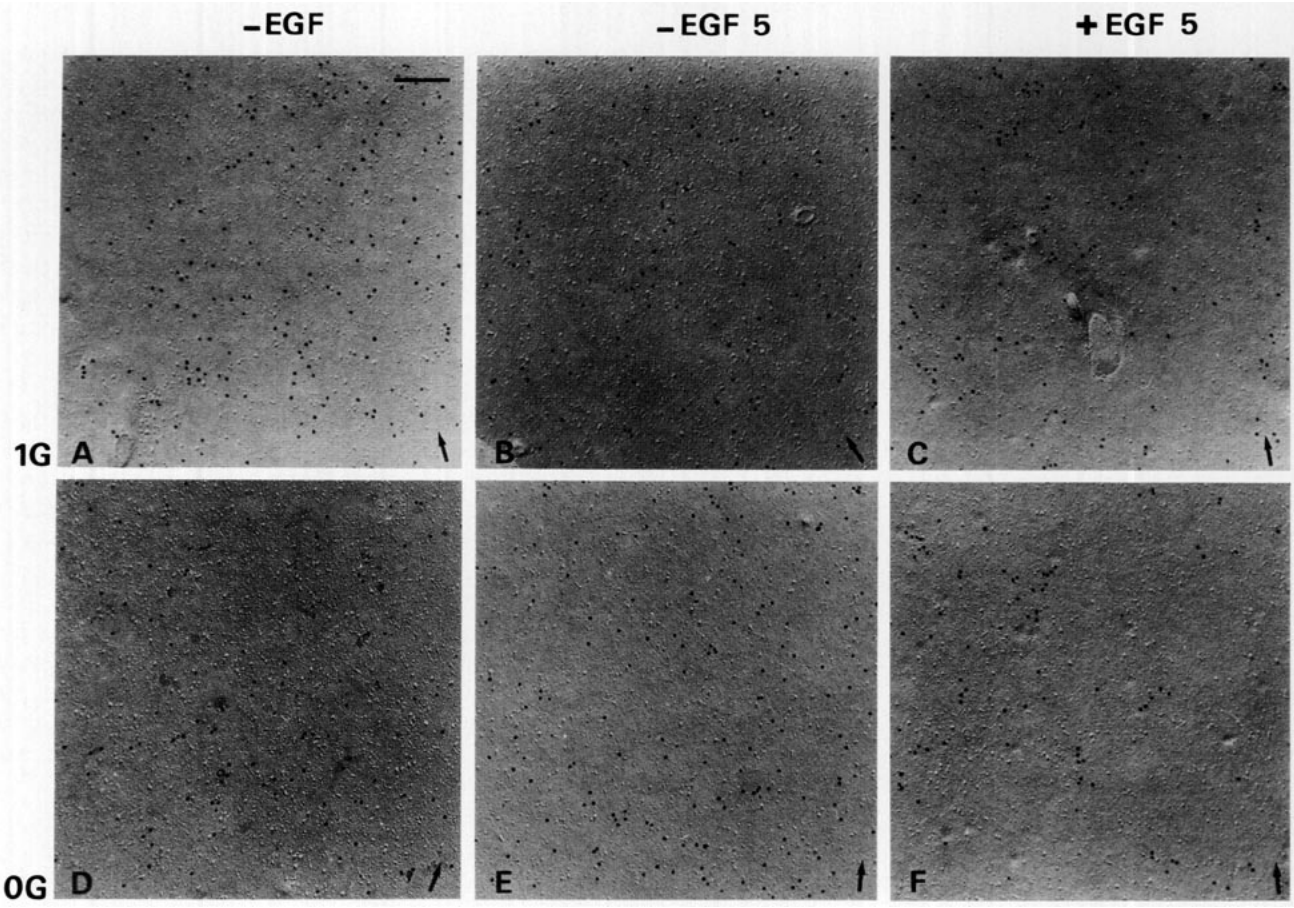

FIG. 4. Qualitative aspects of EGF receptor distribution and IMP distribution in control and EGF-treated A431 cells under different gravity conditions. A431 cells were treated with EGF (80 ng/ml; C and F) or buffer alone (A, B, D, and E) for 5 min under normal gravity conditions (A and D) or in microgravity (B, C, E, and F). Cells were fixed and prepared as described in the legend to Fig. 1. Electron micrographs of the exoplasmic fracture face of cells are shown. Bar indicates 200 nm. Arrows indicate direction of shadowing.

tributions in control or in EGF-treated samples (P = 0.56), as compared to the normal gravity samples.

Normalization of the percentage monomers of normal gravity and microgravity data according to the regression as established for each condition to a particle density of 78 (mean particle density over all experiments) confirms this finding (Fig. 5A). Determination of the Z-value of normal and microgravity receptor distributions also shows that according to this method the receptor distributions of normal gravity and microgravity samples are similar in control and EGF-treated samples (Fig. 5B).

EGF induces increased internalization of EGF receptors [10, 24], possibly leading to a decrease in gold particle density. Apart from receptor redistribution, microgravity may have interfered with the internalization of EGF receptors. However, analysis of variance shows that the average particle density of control and EGF-treated cells is not significantly different after 5 min (Fig. 5C), irrespective of the gravity condition.

In a parallel experiment it was demonstrated that the EGF-induced expression of the c-*fos* and c-*jun* proto-oncogenes in microgravity is suppressed by approximately 50% [7, 8; Table 1], in clear contrast with the effect of microgravity on EGF-induced receptor redistribution. Apparently, the inhibition of EGF-induced c-*fos* expression by microgravity is independent of EGF-induced EGF receptor redistribution. These data sug-

TABLE 1

EGF-Induced c-*fos* and c-*jun* Expression in Microgravity

	1G/0G ratio		
Stimulus	c-*fos*	c-*jun*	β-2 microglobulin
Control	N.D.	N.D.	1.15
EGF	1.9	2.25	0.95

Note. Gene expression was determined by RNase protection analysis, as described [8]. The ratio of gene expression under normal and microgravity, respectively, was determined. N.D., not detectable. As an internal reference β-2 microglobulin expression is determined.

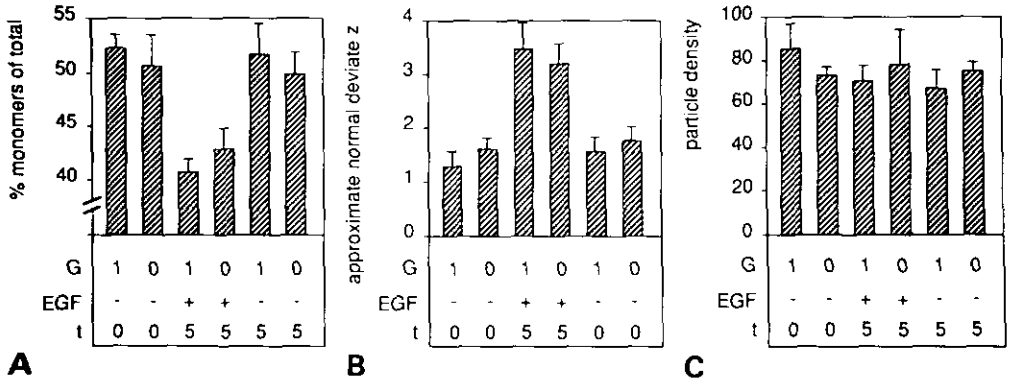

FIG. 5. Effect of microgravity on EGF-induced EGF receptor clustering: a quantitative analysis. A431 cells were brought into microgravity and some samples were fixed at the beginning of the microgravity phase to detect launch effects. Other samples were treated for 5 min with EGF (80 ng/ml) or buffer alone under microgravity conditions. Simultaneously a normal gravity reference experiment was performed. Cells were fixed, immunogold labeled, frozen, and freeze-fractured as described in the legend to Fig. 1. Analysis of receptor distribution was performed according to covariance analysis. (A) The mean percentage monomers of two independent experiments, each consisting of 30 to 40 different samples of launch control (0−), EGF-treated (5+), and control (5−) cells, were normalized, according to the regressions of the percentage monomers on the particle density, as established for control and EGF-treated cells to a particle density of 78 particles per unit area. Data obtained from all normal gravity and microgravity experiments are depicted. (B) Using the same data, the approximate normal deviate Z of the respective gold particle distributions was determined. Z values of launch control (0−), EGF-treated (5+), and control (5−) cells under normal gravity (1*G*) and microgravity (0*G*) are given. (C) The mean particle density of control and EGF-treated A431 cells was determined. The mean particle density of launch control (0*G*−), EGF-treated (5+), and control (5−) cells under normal gravity (1*G*) and microgravity (0*G*) is depicted.

gest that microgravity influences EGF-induced signal transduction downstream of EGF binding and EGF receptor oligomerization, but upstream of the induction of *c-fos* and *c-jun* expression.

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