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Short Communication

CHANGES IN THE CELLULAR DISTRIBUTION OF LIPOCORTIN-1 (ANNEXIN-1) IN C6 GLIOMA CELLS AFTER EXPOSURE TO DEXAMETHASONE

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Abstract—Glucocorticoid-induced changes in cellular levels of Lipocortin-1 (LC-1) (Annexin 1) in C6 glioma cells were determined by electrotransfer and immunoblotting techniques. Separate cell protein fractions were prepared to study the influence of the glucocorticoid steroid, dexamethasone, on LC-1 localisation. Cells were grown in steroid-depleted medium and exposed to dexamethasone (10^{-8} and 10^{-7} M) for 2, 6, and 16 hr. The glucocorticoid-dependent changes in cellular content of LC-1 were both dose- and time-related. Increases above control levels in intracellular and extracellular LC-1 content were detected with the greatest changes occurring at the cell surface. The glucocorticoid-dependent alteration in LC-1 distribution in C6 glioma cells was attenuated by the protein synthesis inhibitor, cycloheximide, indicating the involvement of *de novo* LC-1 synthesis. The significance of these results is discussed in relation to the current concept that some of the anti-inflammatory effect of glucocorticoids occurs through the action of extracellular LC-1.

Key words: C6 glioma cells; glucocorticoid; lipocortin-1; annexin-1

Glucocorticoid steroids have profound anti-inflammatory effects, and influence cell differentiation and proliferation. One mechanism through which glucocorticoids may exert their actions is via the induction of effector proteins termed LCs‡ (for review, see [1, 2]). Much attention has focused on one putative mediator, LC-1, which is a member of the annexin 'superfamily' of calcium- and phospholipid-binding proteins [3]. In addition to the anti-inflammatory actions, members of the annexin family are postulated as being involved in a diverse range of cellular functions, including growth factor signal transduction, exocytosis, and cytoskeletal interactions. LC-1 has been sequenced, and its structure suggests that it is primarily an intracellular protein [4]. However, LC-1 externalisation has been found to occur [5, 6], and extracellular LC-1 shares many of the anti-inflammatory effects of glucocorticoids both *in vitro* and *in vivo* [7–12].

The involvement of LC-1 as a second messenger of glucocorticoid action has been shown in several *in vivo* systems [13–18]. In particular, augmented LC-1 levels associated with infiltrating inflammatory cells have been detected in the spinal cord of rats with EAE, the animal counterpart of MS, whose disease course is regulated by endogenous corticosteroids [19, 20]. In MS and other central nervous system diseases, LC-1 in the target tissues has been found to be associated with reactive astrocytes, the most abundant glia cells in the brain [21–24]. However, the results from *in vitro* studies have been less definite in assigning a role for LC-1. Increases in LC-1 mRNA and total cell LC-1 content have been detected in a variety of cells following glucocorticoid exposure [4, 13, 25–28], although other groups have reported no change occurring in either primary cultures or cell lines [4, 29–31]. Interestingly, recent studies *in vitro* [18, 32] and *in vivo* [33] suggest that glucocorticoids cause changes in the cellular distribution of LC-1 that may not be detectable when total cell content is studied.

The increased presence of LC-1, found by us and others, associated with glia in MS brain tissue, a disease whose pathogenesis can be influenced by glucocorticoids, led us to study the effect of dexamethasone, a synthetic glucocorticoid, on the expression and distribution of LC-1 in the glia-derived C6 cell line.

Materials and Methods

Cell culture. C6 glioma cells (ICN Flow) were used between passage 42 and 52. Culture medium contained Hams F-10 medium supplemented with 2 mM glutamine, 15% horse serum, 2.5% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and fungazone (250 ng/ml) (all from Gibco). Cells were maintained at 37°C in a greater than 90% humidified atmosphere of 5% CO_2/95% air.

Treatment of cultures with dexamethasone alone and in the presence of cycloheximide. Prior to use, confluent cultures were washed with Versene solution, detached using 0.25% trypsin (Sigma), and centrifuged at 120 g for 5 min. Cells were dispensed at 1×10^6 cells/25 cm^2 flask in medium. After 72 hr the cultures were replenished with medium containing serum, which was depleted of steroids by dextran-charcoal treatment. One day later, dexamethasone (cell culture grade, Sigma) (10^{-8} and 10^{-7} M) was added, with ethanol vehicle controls, for either 2, 6, or 16 hr. To determine the involvement of *de novo* synthesis in the action of dexamethasone, the protein synthesis inhibitor cycloheximide (Sigma), dissolved in Hams F-10 medium, was utilised. Initial experiments investigated the influence cycloheximide (5 μg/ml) had on basal LC-1 levels. However, for a direct visual comparison to be made between the dexamethasone-dependent changes in LC-1 in the absence and presence of cycloheximide, subsequent experiments omitted the protein synthesis inhibitor from the controls. In these experiments, cycloheximide (5 μg/ml) was simultaneously added with dexamethasone (10^{-8} and 10^{-7} M) for identical treatment peri-

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‡ Abbreviations: LC, lipocortin; EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; EDTA, ethylenediamine-tetraacetic acid; SDS, sodium dodecyl sulphate.

ods and alterations in LC-1 levels calculated as % changes from control.

Extraction of LC-1 from separate cell fractions. At the termination of the incubation period, both control and treated cells were detached as above, centrifuged at 120 g for 5 min, and cell number adjusted to the same density (2×10^6 cells/ml) for each treatment. Trypan blue exclusion was used to assess cell viability. None of the experimental treatments affected viability. The content of LC-1 in separate cellular fractions was investigated using the method of Peers *et al.* [18] (Fig. 1). The technique allows separation of three fractions: cell-surface, intracellular, and membrane. The intracellular fraction contained predominantly cytoplasmic protein. The membrane fraction comprised membrane/cytoskeleton and mitochondria. Samples were stored in 10 mM EDTA to minimise the precipitation of LCs from calcium-containing solutions.

All fractions, extracted from the same number of cells (2×10^6 cells/ml), were prepared in the same final volume to facilitate a direct comparison of relative abundance of LC-1 by Western blotting.

Detection of LC-1. Samples were boiled for 5 min in buffer containing 2% SDS and 5% 2-mercaptoethanol (BDH) and applied to 1.5 mm, 10% polyacrylamide minigels. SDS-polyacrylamide gel electrophoresis was performed by conventional methods [34], the samples were transferred electrophoretically onto nitro-cellulose sheets, and LC-1 detected using a sheep antibody directed against human recombinant LC-1 (a gift from Dr. J. D. Croxtall). LC-1 was visualised by the deposition of peroxide-oxidised diaminobenzidine following incubation with peroxidase-linked donkey anti-sheep antibody (IgG) (Sigma).

Quantitation of LC-1 band staining intensity was undertaken by volume analysis using a BIO-RAD GS-670 densitometer. Results were calculated as % change from vehicle control run on the same gel.

Results

Basal distribution of LC-1. Control levels of LC-1 present in each of the fractions was quantitated by densitometric comparison with a known quantity of LC-1 standard. The intracellular fraction contained 256 ± 38 ng of LC-1, and the membrane and cell-surface values were 28 ± 4 ng and 3 ± 2 ng LC-1, respectively ($n = 6$).

Time- and dose-dependent effect of dexamethasone on cellular distribution of LC-1. Dexamethasone at concentrations of 10^{-8} and 10^{-7} M was added to cultures for 2, 6, and 16 hr and changes in the cellular distribution of LC-1 measured. Figure 2(a(i)–c(i)) shows the mean % changes from controls for each time and dose on three cell fractions: cell-surface, membrane, and intracellular. Glioma cells in the presence of 10^{-8} M dexamethasone for 2 hr showed minimal change in LC-1 content in all fractions. After 6 hr treatment with low-dose dexamethasone, increases in LC-1 were detected in cell-surface and membrane fractions. The increased LC-1 present at the cell surface remained high following incubation with dexamethasone for 16 hr.

Figure 2 (d(i)) demonstrates that in the presence of the higher concentration of dexamethasone (10^{-7} M) for 2 hr, C6 glioma cells respond by increasing LC-1 content associated with the membrane, with no change occurring in the other cell fractions. Following 6 hours of glucocorticoid treatment, a substantial rise in extracellular LC-1 was apparent, the levels of which did not alter after further exposure (16 hr) to dexamethasone (Fig. 2 (e(i) and f(i)). Comparison of the increases in cell-surface LC-1 induced by 10^{-8} M and 10^{-7} M dexamethasone suggests the response is dose-related.

Effect of cycloheximide on dexamethasone-induced changes in LC-1. Cycloheximide (5 μg/ml), an inhibitor of *de novo* protein synthesis, reduced basal LC-1 levels in all cell fractions

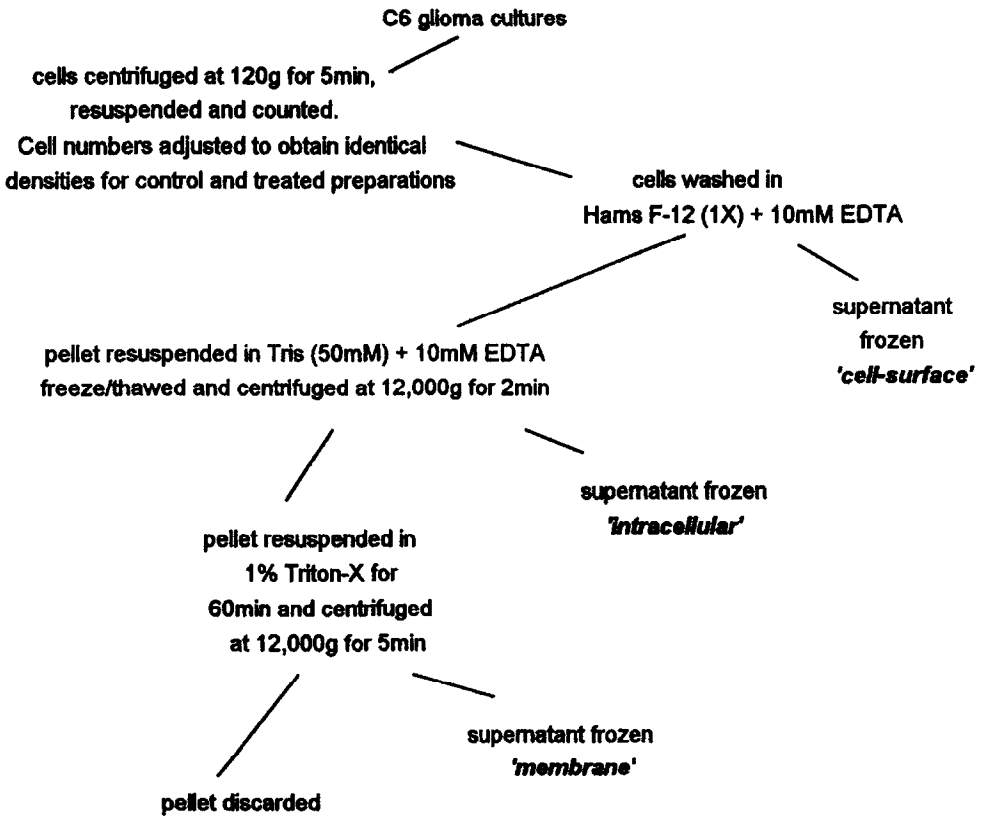

Fig. 1. Method for obtaining separate cell fractions.

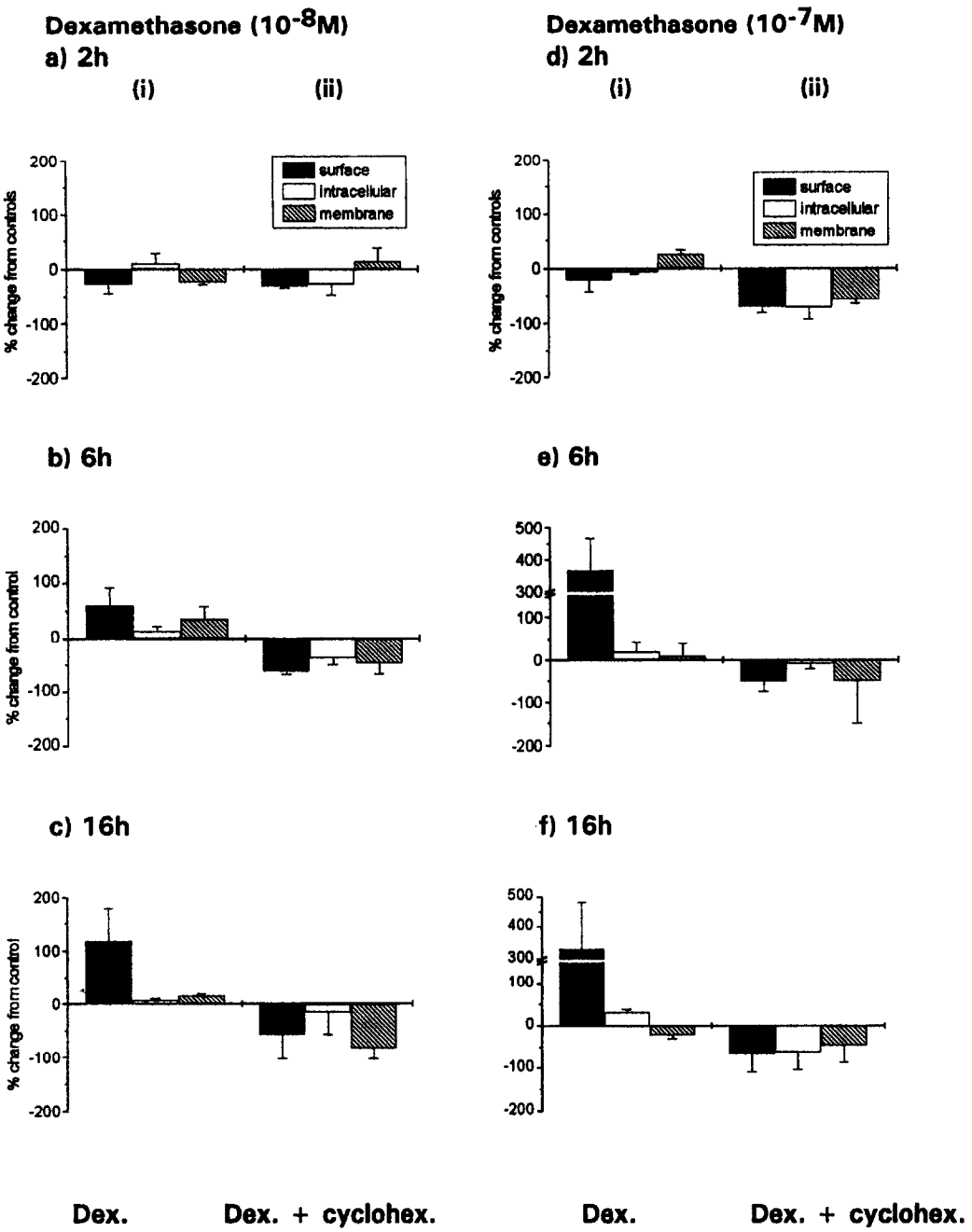

Fig. 2. Comparison of the changes in cellular location of LC-1 after dexamethasone treatment in the (i) absence and (ii) presence of the protein synthesis inhibitor, cycloheximide. Values represent mean data (±*SEM*) of % changes from control after densitometric analysis of immunoblots (3–6 separate experiments). Each fraction (control and dexamethasone-treated) was derived from the same number of cells. Percentage changes in LC-1 associated with the cell surface, membrane, and intracellular fractions following treatment of cell cultures with dexamethasone (10^{-8} M) for (a) 2 hr, (b) 6 hr, and (c) 16 hr. Changes after treatment with dexamethasone (10^{-7} M) for (d) 2 hr, (e) 6 hr, and (f) 16 hr. At each dose and time point the effect of the protein synthesis inhibitor, cycloheximide, was assessed: (i) Dex. (dexamethasone alone), (ii) Dex. + cyclohex. (dexamethasone in the presence of cycloheximide, 5 μg/ml).

(−71 ± 6% of control; mean value of all fractions, 16 hr), indicating that *de novo* synthesis is involved in the basal occurrence of LC-1, which is unrelated to glucocorticoid exposure. Figure 2 (a(ii)–f(ii)) shows that concurrent addition of cycloheximide with dexamethasone (10^{-8} and 10^{-7} M) abolished glucocorticoid-related changes in LC-1 associated with all cell fractions.

Discussion

A comparison of the amounts of LC-1 associated with the extracellular, membrane, and intracellular fractions under basal conditions reveals that the greater proportion of cell LC-1 is present intracellularly. The results are in agreement with our findings in primary astrocyte cultures [35], and with the results

of Peers *et al.* [18] in rat peritoneal leukocytes. The high basal levels of LC-1 within cells under steroid-free conditions indicate that LC-1 synthesis can be modulated by additional factors unrelated to glucocorticoids. Support for this suggestion is provided by the studies of Browning *et al.* [4] showing that the LC-1 gene contains response elements that can be regulated by non-glucocorticoids. The basal presence of LC-1 may also be accounted for by the widely reported involvement of the protein in cytoskeletal-membrane interactions [36–38] and cell growth and differentiation [2, 39, 40].

Our studies also describe the time- and dose-dependent changes in LC-1 associated with different cellular fractions after exposure of C6 glioma cells to the glucocorticoid dexamethasone. The cellular distribution of LC-1 in C6 glioma cells was altered following treatment with the steroid. An increase in LC-1 content was observed at the cell surface 6 hr after glucocorticoid (10^{-8} M) treatment, with a further enhancement at 16 hr. The changes occurred in conjunction with lesser rises in LC-1 associated with the intracellular and membrane fractions, and are in contrast to previous studies in a variety of established cell lines where levels of LC-1 did not alter [4]. One possible explanation for the disparity in results is that in previous investigations, total cell LC-1 was observed, rather than the more subtle changes in separate cell fractions. Therefore, any changes in distribution within the cell and also any externalisation of LC-1 may not be apparent.

In the current study, the actions of dexamethasone on LC-1 distribution were dose- and time-related. To elucidate the contribution made by *de novo* synthesis in the dexamethasone-dependent effect, the protein synthesis inhibitor cycloheximide was used. Concomitant incubation of dexamethasone-treated cells with cycloheximide totally inhibited steroid-induced changes in LC-1 at all time points. Moreover, basal LC-1 levels were also attenuated. These results suggest that the alterations in LC-1 content that occur upon dexamethasone treatment are dependent on *de novo* protein synthesis. In addition, *de novo* synthesis appears to contribute to basal LC-1 expression. Early work by Blackwell *et al.* [41] also showed that hydrocortisone-induced release of LC from peritoneal macrophages was inhibited by cycloheximide.

The structure of LC-1 suggests that it is primarily an intracellular protein. It lacks both glycosylation and a hydrophobic signal sequence [26, 42, 43], and is acetylated at the amino terminus [44]. However, Pepinsky *et al.* [45] found low amounts of LC-1 in peritoneal exudates after glucocorticoid treatment, leading to the first suggestion that LC-1 is secreted and may have an extracellular role. Subsequent studies have reported that the secretion of LC is a physiological process and not a result of nonspecific release from dead or damaged cells [5, 6]. However, the extracellular presence of LC-1 is not detected in all cell systems [30, 46, 47]. The mechanism by which LC-1 is transported out of the cell is not understood, since it lacks the required signaling sequence for externalisation. LC-1 is not unique in having an extracellular presence but lacking the requisite hydrophobic signalling structure. Other factors in this category are interleukin-1 [48, 49] and fibroblast growth factor [50, 51]. The ability to detect glucocorticoid-induced increases in extracellular LC-1 in some cell systems lends support to the currently held view that LC-1 may exert its biological actions through cell-surface binding sites, and this concept has been substantiated by several findings both *in vitro* and *in vivo* [7–10, 12]. The results described in the current study indicate that in C6 glioma cells dexamethasone affects the distribution of LC-1 intracellularly and extracellularly. This suggests that LC-1 has functions at more than one cellular locality.

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