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Glucocorticoid Dexamethasone Reversibly Complements EJ-RAS Oncogene to Transform Mouse Embryo BALB-3T3 Cells

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EJ-A is a Balb-3T3 transfectant cell line that bears a small number of EJ-ras oncogene copies/cell, has low EJ-ras expression, and resembles the parental cell line in displaying a non-transformed phenotype. The glucocorticoid hormone dexamethasone reversibly induces transformation traits in EJ-A cells, namely: 1) morphological transformation; 2) increased growth rate and saturation density; 3) reduced G_1 length; and 4) independence of the FGF requirement to initiate DNA synthesis. Western blot analysis revealed that dexamethasone does not increase the $p21^{ras}$ protein intracellular level. β-IFN, added to the culture medium, does not suppress the dexamethasone-induced growth stimulation and morphological transformation. Therefore, glucocorticoid hormones can complement low EJ-ras expression to transform Balb-3T3 cells, by a mechanism that is likely to be independent of $p21^{ras}$ increase and β-IFN decrease.

Key words: EJ-ras oncogene transfectant, glucocorticoid hormone, reversible transformation, $p21^{ras}$, β-IFN

Reports from several laboratories described a variety of effects of glucocorticoid hormones upon cell-cycle regulation [1–7]. More recently, it has been shown that glucocorticoids inhibit synthesis of peptide growth factors and interferon [8–12], hence, modulating cell growth and possibly expression of the transformed phenotype.

In this paper, we report that dexamethasone reversibly complements the transforming activity of mutated human c-Ha-ras-1 oncogene (EJ form) in Balb-3T3 mouse embryo cells. The mechanism underlying this dexamethasone effect does not seem to involve increased EJ-ras expression or inhibition of endogenous β-IFN synthesis.

MATERIALS AND METHODS

Cells

The original stock of early passage Balb-3T3, clone A31, came from Dr. Charles D. Stiles laboratory (Dana-Farber Cancer Institute, Boston). Cultures were maintained under strict regimen of cultivation: continuously growing from sparse to near confluent

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and subcultured every 3–4 days. The age of a culture, estimated from the number of population doublings, was carefully registered. Cultures were grown in Dulbecco's modified Eagle's medium (DME) plus 1.2 g/l sodium bicarbonate and 10% fetal calf serum (FCS). Culture flasks were flushed with 5% CO_2—95% air and sealed; plates were maintained in plastic boxes flushed with the same gas mixture.

EJ-ras Transfectants

A series of Balb-3T3 transfectants were previously obtained [13] by DNA-mediated transfections with the human EJ-ras plasmid pEJ6.6 from Dr. R. Weinberg's laboratory, MIT [14]. This series comprised cell clones with variable oncogene dosage per cell. The EJ-A transfectant clone, used in this work, carries less than five EJ-ras copies per cell and displays low expression of this oncogene [13].

Growth Curves

In 10% FCS-DME, 5×10^4 cells were plated per 35 mm-diameter dish. Five to six hours later, dexamethasone and/or IFN were added to some dishes. Duplicate plates were collected daily for 7–9 days, and the cells were trypsinized, formaldehyde-fixed, and counted in an electronic cell counter. Culture medium was changed every other day. Doubling times were estimated from the slope of the best-fitted straight line to the first four points of the curves in semilog plots. Saturation densities were derived from plateau values in the curves.

[^{3}H]Thymidine Incorporation and Autoradiography

In 10% FCS-DME, 5×10^3 cells were plated per 0.9 cm^2 coverslip. Forty-eight hours later, the medium was changed to serum-free DME for 36 h. Cells were then restimulated to initiate DNA synthesis by addition of serum or growth factors to the medium. [^{3}H]thymidine (0.25 μCi/ml; 10^{-7} M) was incorporated for the indicated time and autoradiography performed as previously described [15]. To estimate the lag before the onset of DNA synthesis (G_1 phase length), FCS, dexamethasone, and [^{3}H]thymidine were added at zero time and two coverslips were collected per point every other hour for 26–30 h. The lag was estimated from graphs of nuclear labeling index versus time in hours.

Western Blotting [16]

Cells were lysed in 1% Triton X-100 and 2% β-mercaptoethanol in PBS. Two hundred micrograms total cellular protein per lane were fractionated in 12% polyacrylamide-SDS slab gels and electrotransferred (200 V for 15 h) to 0.2 μm nitrocellulose membranes (S & S BA 83). Following transfer, the membranes were stained with 0.1% Ponceau, photographed to monitor the amount of protein actually transferred, and, to estimate migration of molecular weight standards, incubated in blocking solution (5% non-fat milk in PBS) before antibody reaction. Monoclonal antibody against a Harvey v-ras synthetic peptide (Hibridioma no. 142-24ES from Microbiological Associates) was used at 1/200 dilution in blocking solution. Antibody binding was assessed using freshly iodinated [^{125}I]protein A (1.5×10^6 cpm/ml) diluted in blocking solution. After a thorough wash, the membranes were exposed to Kodak XK-1 film.

Tumorigenicity Assay

After trypsinization, 10^6 cells were suspended in 0.1 ml 2% FCS-DME and injected subcutaneously into the scapular region of 6 week old immunocompetent syngeneic Balb-c mice. The animals were examined daily for palpable nodules to determine the latent time before tumor onset; animals were only scored as positive when the initial palpable nodule developed into a large tumor. Animals without tumors were kept for 2.5 months before being sacrificed.

RESULTS AND DISCUSSION

EJ-A cells [13] are low-expression transfectants of the EJ-ras oncogene (less than five copies/cell) that resemble the parental Balb-3T3 cell line in displaying a non-transformed phenotype (stage I cells). Upon serial cultivation, stage I EJ-A transfectant cells spontaneously undergo progressive transformation (without EJ-ras oncogene amplification and/or overexpression), reaching a fully transformed state (stage III cells) [13].

Dexamethasone treatment leads to the appearance of several transformation traits in stage I EJ-A cells, namely: 1) increased growth rate and saturation density (Table I); 2) piling-up with abundant criss-crossing in confluent cultures (Fig. 1A,B); and 3) reduction in the lag before DNA synthesis initiation (G_1 phase) in Go-arrested stage I EJ-A cells, growth restimulated by serum refeeding (Table I). None of these dexamethasone effects was observed with the parental Balb-3T3 cells (Table I; Fig. 1). Stage III EJ-A cells, which already display a transformed phenotype [13], were not affected by the glucocorticoid hormone (Table I; Fig. 1).

Dexamethasone overides the requirement for competence growth factors to initiate DNA synthesis in serum-free medium displayed by stage I EJ-A cells (Fig. 2). The same growth factors are required by stage I EJ-A and parental Balb-3T3 cells, namely, a combination of FGF, EGF, and insulin (Fig. 2A,B). Dexamethasone alone did not

TABLE I. Effects of Dexamethasone on Growth of Parental Balb-3T3 Cells and Stages I and III EJ-A Transfectants

Growth parameter	Cell line	Control (untreated)	Dexamethasone treated
Doubling time (h)	Parental Balb-3T3	19.5(1)	19.5(1)
	EJ-A-I	21.9 ± 0.8(3)	16.9 ± 1.0(3)
	EJ-A-III	13.5(1)	13.5(1)
Saturation density (cells/cm^2 × 10^5)	Parental Balb-3T3	0.65(1)	0.65(1)
	EJ-A-I	1.7 ± 0.8(3)	3.2 ± 0.6(3)
	EJ-A-III	4.0(1)	[a]
G_1 length (h)	Parental Balb-3T3	12(3)	12(1)
	EJ-A-I	12(3)	10(1)
	EJ-A-III	6(3)	ND

ND: Not done. Nos. in parenthesis indicate the number of independent experiments. Figures are averages ± s.e.m.

[a]Determination imprecise because of cell detachment upon confluency caused by the hormone treatment.

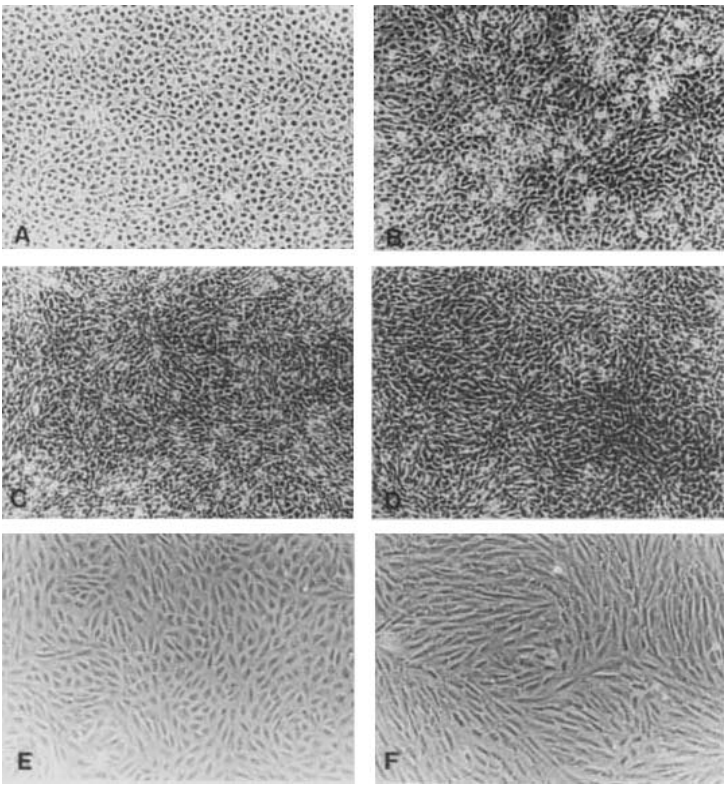

Fig. 1. Dexamethasone effects on morphology of stages I and III EJ-A transfectants and parental Balb-3T3 confluent cultures. **A,B:** Stage I EJ-A cells untreated and treated with dexamethasone; **C,D:** stage III untreated and treated; **E,F:** parental Balb-3T3 untreated and treated, respectively. Cells were plated at low density in 10% FCS-DME with and without dexamethasone (10^{-7} M) and grown to confluency. Photomicrographs were taken under a phase contrast microscope (100× magnification) at day 7.

stimulate DNA synthesis (Fig. 2A,B). As expected, the hormone caused an enhancement effect on DNA synthesis stimulation by FGF plus insulin for both cell lines (Fig. 2A,B). However, dexamethasone combined with insulin, had a large synergistic effect on DNA synthesis stimulation of EJ-A transfectants (Fig. 2B) but not of parental cells (Fig. 2A). Therefore, dexamethasone mimics a competence growth factor for stage I EJ-A cells. These observations are particularly important, because when EJ-A transfectants change from stages I to III, the cells lose their requirement for FGF and PDGF [13]. Moreover, this change correlates with acquisition of maximum tumorigenic potential in immunocompetent syngeneic mice [13].

Maximal dexamethasone effect was obtained at 10^{-7} M whereas 10^{-8} and 10^{-9} M were, respectively, suboptimal and uneffective. The effect is completely reversible: Stage I EJ-A cells treated with dexamethasone (10^{-7} M) for 7 days and then replated in dexamethasone-free medium displayed the same growth rate, saturation density, and morphology of untreated control cultures (not shown). Furthermore, dexamethasone pre-treatment of stage I EJ-A cells did not significantly increase its tumorigenic potential in immunocompetent syngeneic mice (Table II). Table II also shows that stage III EJ-A cells, obtained from stage I cells by serial cultivation (see [13]), are highly tumorigenic.

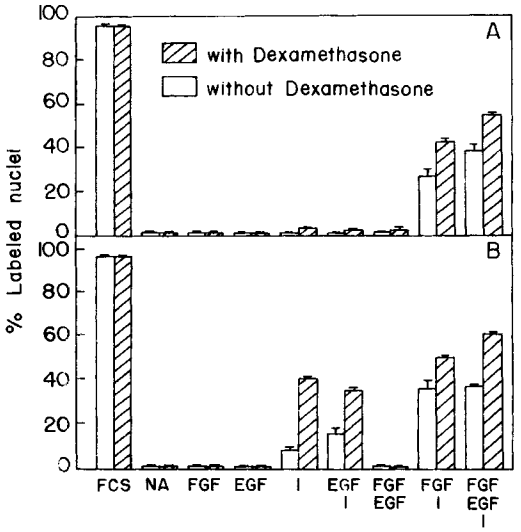

Fig. 2. Restimulation of DNA synthesis initiation in cell cycle arrested (stage I) EJ-A transfectant and Balb-3T3 parental cells: Effect of dexamethasone on growth factors' activity assayed by autoradiography. **A:** Balb-3T3 parental cells; **B:** stage I EJ-A transfectants. NA: control, no addition. FGF (1 ng/ml): pure bovine pituitary basic fibroblast growth factor, prepared by Dr. A.G. Gambarini and P.L. Ho in this laboratory; EGF (10 ng/ml): pure epidermal growth factor from Collaborative Research, Boston, MA, USA: I (1 μg/ml), pure insulin from Sigma, St. Louis, MO.

TABLE II. Tumorigenic Potential of EJ-A Transfectants Upon Dexamethasone Pre-treatment

Cell line	Dexamethasone pre-treatment	Animals with tumor/ injected animals	Latent period (days)
EJ-A-I[a]	–	0/5	–
	+	1/5	30
EJ-A-III	–	4/4	14–23
Early passage parental Balb-3T3	–	0/5	–
Late passage parental Balb-3T3[b]	–	0/5	–

[a]Cells grown to confluency in medium containing 10^{-7} M dexamethasone for 7 days and kept in dexamethasone-free medium for 3 days before injections; untreated controls were parallel cultures maintained under the same regimen but in the absence of dexamethasone.

[b]Early passage are morphologically normal cells of low saturation density (0.5–0.6 $\times$ 10^5 cells/cm^2) and late passage (more than 6 months continuous cultivation) are morphologically transformed-like cells of high saturation density (>4 $\times$ 10^5 cells/cm^2).

However, long cultivation periods are not sufficient to render the parental Balb-3T3 cells tumorigenic (Table II).

We described elsewhere [13] that EJ-A cells (stages I–III) are low-expressing transfectants of the EJ-ras oncogene. Here we show, by Western blot analysis, that dexamethasone does not alter EJ-ras expression in either stage I or III cells (Fig. 3).

TABLE III. Effects of β-IFN and Dexamethasone on Growth of Stage I EJ-A Cells

Additions to medium	Doubling time (h)	Saturation density (cells/cm^2 × 10^5)
Control (no additions)	24.0	1.2
β-IFN (50 U/ml)	32.2	1.3
Dexamethasone (10^{-7} M)	19.4	2.4
β-IFN plus dexamethasone	24.0	2.5

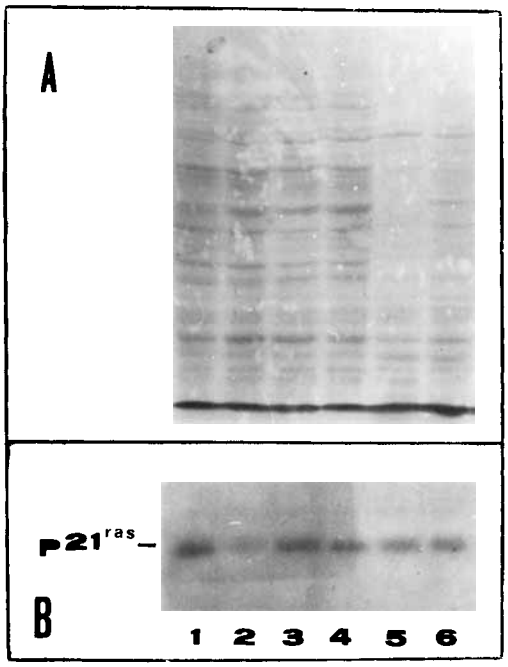

Fig. 3. Intracellular levels of p21^{ras} protein in cells untreated and treated with dexamethasone, assessed by Western blotting with a monoclonal antibody against a Harvey v-ras synthetic peptide. **A:** Nitrocellulose membrane stained with Ponceau. **B:** Autoradiogram after ^{125}I-protein A reaction. 1,2: Parental Balb 3T3 cells untreated and treated with dexamethasone; 3,4: stage I EJ-A cells untreated and treated; 5,6: stage III EJ-A cells untreated and treated, respectively. Exponentially growing cells were treated with dexamethasone (10^{-7} M) for 48 h before lysis; for other details see Materials and Methods.

Therefore, the transforming effect of dexamethasone on stage I cells is not due to an increase in p21^{ras} intracellular level.

It has been reported that β-IFN blocks PDGF stimulation of Balb-3T3 cell growth [17]. Also, it has been proposed [11] that dexamethasone enhances growth-factor activity on human diploid fibroblasts by suppressing the growth-factor-induced β-IFN production.

In fact, murine β-IFN, in the range of 50–150 U/ml, inhibited growth of both parental Balb-3T3 and EJ-A transfectant cells (stages I–III). The doubling time was increased by 30–40%, but no difference in saturation densities was detected for long-term cultures (8–9 days). In spite of its growth inhibitory effect, β-IFN did not suppress the dexamethasone-induced growth stimulation (Table III) and morphological transfor-

mation (Fig. 1) of stage I cells. Table III shows that β-IFN caused an increase in stage I EJ-A cells' doubling time from 24 to 32.2 h; addition of dexamethasone to cultures stimulated growth, reducing the doubling time to 19.4 h, whereas combination of β-IFN and dexamethasone led to a doubling time of 24 h. β-IFN caused no change in saturation density; dexamethasone, on the other hand, led to a twofold increase, irrespective of β-IFN presence. These results suggest that the mechanism by which dexamethasone induces transformation-like changes in stage I EJ-A cells is not via inhibition of IFN endogenous synthesis.

Neoplastic transformation of primary rat embryo cells by EJ-ras oncogene requires co-transfection of multiple oncogenes [18–21]. This paper shows that the phenotype of Balb-3T3 EJ-ras transfectants can be modulated by a hormone. These two approaches [i.e., 1) definition, by DNA-mediated transfection, of complementing oncogenes and 2) identification of hormones that modulate the transformed phenotype of oncogene transfectants] concur to elucidate the regulatory circuitries that control cell growth.

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