EXPERIMENTAL STUDY

Association between the expression of E1A oncogene and increased sensitivity to growth inhibition induced by sustained levels of cAMP in rat thyroid cells

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Abstract

Objective: The aim of this study was to investigate: (i) whether a persistent increase of cAMP interferes with the proliferation of transformed thyroid cells, and (ii) whether the degree of malignancy is correlated with the sensitivity to a transient and/or sustained increase in intracellular cAMP levels.

Design and methods: To address these questions we used thyroid cell lines transformed with E1A oncogene from adenoviruses 5 (PC E1A cell line) or 2 (PC HE4 cell line), or infected with the polyoma murine leukemia virus (PC PyMLV cell line) carrying the middle T gene of the polyoma virus, or, finally, expressing both E1A and PyMLV. These cell lines present various degrees of malignancy: PC E1A and PC HE4 cells are not tumorigenic; PC PyMLV cells induce non-invasive tumors after a long latency period; and PC E1A + PyMLV cells are highly tumorigenic.

Results and conclusions: Thyroid cell proliferation required the transient increase of intracellular cAMP levels, while persistent elevation of cAMP blocked the proliferation of normal thyroid PC Cl 3 cells and of PC Cl 3 cells transformed by a variety of different oncogenes. In addition, sustained levels of cAMP induced apoptosis in cells carrying the adenovirus EIA oncogene, but not in cells transformed with other oncogenes or in the wild-type PC Cl 3 cells. Furthermore, middle T gene of the polyoma virus seemed to afford protection only from apoptosis induced by cAMP when middle T is present in thyroid cells along with the E1A gene.

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Introduction

The duration of a given stimulus seems to play a key role in the regulation of cellular responses. Persistent versus transient activation of certain molecules can be used by the cellular machinery to elicit diverse cellular responses. The same growth factor may regulate differing and even opposite cellular responses, by inducing transient or persistent activation. In PC 12 cells, for example, it has been clearly demonstrated that regulation of cell proliferation or differentiation is determined by the duration of extracellular signal-regulated kinase activation (1). Recently, Scharemberg & Kinet (2) analyzed the key role of sustained inositol trisphosphate levels on the homeostasis of intracellular calcium and the differing responses elicited by transient and sustained intracellular calcium levels. The temporal characteristics of the calcium signal, including whether the signal is sustained or transient or oscillatory, can be important determinants of the activation of specific transcription factors and so of the type of transcriptional response that occurs (2).

Thyroid cells are the biological targets of thyrotropin (TSH), which exerts its biological effects via the adenylate cyclase/cAMP system (3–8). TSH induces and sustains the proliferation of rat thyrocytes FRTL5 and PC Cl 3 (9, 10). The increase of intracellular cAMP induced by TSH in thyroid cells is very rapid but transient because TSH also stimulates cAMP-dependent phosphodiesterases, which cause cAMP degradation (11, 12).

We have recently reported (13) that the transient nature of intracellular levels of cAMP is a key step in the regulation of FRTL5 cell proliferation; if cells are treated with phosphodiesterase inhibitors, their proliferation is inhibited in the presence of TSH. Thus, although the increase in intracellular cAMP levels is necessary to induce thyroid cell proliferation, degradation of cAMP is also important.
The purpose of this study was to investigate whether a persistent increase of cAMP interferes with the proliferation of transformed thyroid cells and whether the degree of malignancy is correlated with the sensitivity to transient or/and sustained increase in intracellular cAMP levels.

To address these questions we used thyroid cell lines transformed with E1A oncogene from adenoviruses 5 (PC E1A cell line) or 2 (PC HE4 cell line), or infected with the polyoma murine leukemia virus (PC PyMLV cell line) carrying the middle T gene of the polyoma virus, or, finally, expressing both E1A and PyMLV (10–12). These cell lines present various degrees of malignancy: PC E1A and PC HE4 are not tumorigenic; PC PyMLV cells induce non-invasive tumors after a long latency period; and PC E1A+PyMLV cells are highly tumorigenic.

TSH increased the growth rate of PC E1A, PC HE4 and PC PyMLV cells. Furthermore, sustained levels of cAMP influenced the proliferation of PC E1A, PC HE4, PC PyMLV and PC E1A+PyMLV cells, as well as of PC Cl 3. In addition, sustained levels of cAMP increased the apoptotic response only in the cells carrying the E1A oncogene.

Materials and methods

Materials

Materials were purchased from the following sources: Coon’s modified Ham F12 tissue culture medium (mF12) from Sigma Chemical Co. (St Louis, MO, USA); calf serum from Gibco (Grand Island, NY, USA); BSA from Reheis Chemical Co. (Phoenix, AZ, USA); tissue culture dishes (100 mm) and 60 mm) and centrifuge tubes (15 ml and 50 ml) from Falcon, Beckton, Dickinson and Co. (Oxnard, CA, USA); 24-well culture plates from Costar (Cambridge, MA, USA); isobutyl methylxanthine (IBMX), bovine TSH (bTSH) and Kemptide from Sigma Chemical Co; Triton X-100 and Bio-Rad Protein Assay from Biorad Laboratories (GmbH, Munich, Germany); RNAase from Fluka Chemie AG (Buchs, Switzerland); [methyl-3H]thymidine (1.0 mCi/ml) and [γ-32P]ATP (10 mCi/ml), from Amer sham International (Amer sham, Bucks, UK). Highly purified bTSH was kindly provided by the National Hormone and Pituitary Program, NIDDK, NIH, University of Maryland School of Medicine, MD, USA.

Culture techniques

PC Cl 3 and PC E1A, PC HE4, PC PyMLV and PC E1A+PyMLV cell lines were used in this study. Cells were cultured in mF12 medium supplemented with 5% calf serum, in 100-mm tissue culture plates at 37°C in an atmosphere of 95% air – 5% CO2 in a humidified incubator. The medium was supplemented with a mixture of six hormones and growth factors: bTSH (1 mU/ml), insulin (1 mg/ml), transferrin (5 mg/ml), hydrocortisone (3.62 ng/ml), somatostatin (10 ng/ml), and glycy-histidyl-lysine (10 ng/ml) (6H medium). In some studies, cells were maintained without bTSH and the other five hormones (H-free medium).

Growth curve

Cell proliferation was determined by growth curve analysis, as previously described (13). Cells were seeded in mF12 medium supplemented with 5% calf serum and 6H mix with and without 1 mM IBMX at a concentration of 2.5 × 10^5/60 mm culture dish. After 7 days of treatment with TSH and IBMX, IBMX was removed. At each experimental point triplicate dishes were trypsinized and the cell number was determined by counting cell suspensions in a Neubauer hemocytometer.

Measurements of cAMP concentration

The effects of concomitant treatment with TSH and IBMX on cAMP generation were measured as previously described (13). Cells were grown to confluence in 6H medium in 24-well Costar plates and then maintained in H-free medium for 5 days. Monolayers were washed three times with Krebs–Ringer buffer containing 0.1% BSA, pH 7.2, and cells were then incubated at 37°C in 250 μl of the same buffer containing the appropriate concentrations of the agent to be tested. After 30 min, the supernatants were collected and frozen at −20°C. To extract intracellular cAMP, monolayers were treated overnight at −20°C with 500 μl ice-cold absolute ethanol. Extracts were then dried and each pellet was reconstituted with its original supernatant. Aliquots of appropriate volume were taken for RIA of cAMP concentrations. Equivalent wells were employed for determination of cell numbers and results were expressed as pmol cAMP/10⁶ cells.

DNA fragmentation analysis

After 10 h of treatment, the cells were trypsinized and counted, then 2 × 10⁶ cells per sample were lysed in 0.5% Triton X-100, 5 mM Tris buffer pH 7.4, 20 mM EDTA for 20 min at 4°C. After centrifugation at 20000 × g in a microfuge (Eppendorf 5415C; Eppendorf-Netheler-Hinz GmbH-22 331, Hamburg, Germany), supernatants were extracted with phenol–chloroform and precipitated in ethanol. Soluble DNA was resuspended in 10 mM Tris, 1 mM EDTA buffer, and incubated with 50 μg/ml RNAase A for 3 h. All the soluble DNA recovered per sample was electrophoresed on a 1.2% agarose gel and stained with ethidium bromide.

Assay of cAMP-dependent protein kinase activity

Cell monolayers in 60-mm tissue culture dishes were washed twice with cold PBS and kept on ice. Cells were removed using a rubber policeman with 4 ml
ice-cold PBS, then centrifuged for 10 min at 1500 r.p.m. The cell pellets were suspended in 60 μl of a solution containing 20 mM Hepes, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 20% glycerol, 50 mM NaCl and 1% Triton, and kept for 10 min on ice. Cell extracts were then centrifuged at 4°C for 20 min in the microfuge. Protein content was determined by the Biorad assay, and 2.5 mg protein were incubated for 10 min at 30°C with a solution containing 100 mM \([^{32}P]\)ATP (125–150 c.p.m./pmol), 5 mM MgAc, 15 mM Kemptide, 10 mM Tris–HCl (pH 7.4), 250 mM IBMX, 5 mM DTT, 2.5 mM NaF in a final volume of 25 μl; where indicated, 5 mM cAMP were added. Kemptide phosphorylation was monitored by spotting 20 μl of the incubation mixture on phosphocellulose filters (Whatman P81; Whatman Ltd, Maidstone, UK). Filters were washed with 75 mM phosphoric acid as previously described (13), and counted in a beta scintillation counter. The assay was performed in quadruplicate for each experimental point. The results are expressed as pmol radiolabeled substrate/μg protein over 10 min of incubation in the presence or absence of cAMP.

Results

Effect of sustained levels of cAMP on the proliferation of normal and transformed thyroid cells

PC Cl 3 cells proliferated only in the presence of TSH as already reported (10). PC E1A, PC HE4, PC PyMLV and PC E1A + PyMLV proliferated also in the absence of this hormone (14).

The concomitant presence of TSH and IBMX in the culture medium completely blocked cell proliferation of both normal and transformed cells. Cell proliferation was restored upon withdrawal of IBMX from the culture medium (Fig. 1). It is noteworthy that, while an association of 1 nM TSH and 1 mM IBMX blocked cell proliferation in cell lines carrying E1A this treatment caused a remaining substantially unchanged for 7 days (Fig. 1), in cell lines carrying E1A this treatment caused a dramatic decrease in the cell number: from 2.5 × 10^5 at the day of plating to 2.7 × 10^4 at day 7 (Fig. 1). When E1A was expressed in thyroid cells in combination with PyMLV, the decrease in cell number, although still consistent, was less dramatic than in the former case: from 2.5 × 10^5 at the day of plating to 5.9 × 10^4 at day 7 (Fig. 1).

To demonstrate that the inhibitory effect of sustained levels of cAMP on the proliferation of PC E1A cells was a general phenomenon due to the presence of E1A and not to a peculiarity of PC E1A cells, a cell line transfected with the E1A gene from adenovirus 2 instead of 5 (PC HE4 cells) was tested. The concomitant TSH and IBMX treatment induced a decrease also in the number of PC HE4 cells superimposable on that observed in the PC E1A cells (data not shown).

Sustained levels of cAMP can induce apoptosis in E1A-expressing cells

We used the DNA fragmentation assay to test whether cell death caused by IBMX and TSH treatment correlated with the activation of an apoptotic process. PC Cl 3 cells did not present DNA fragmentation in any of the conditions tested (Fig. 2, lanes 1, 2 and 3). PC PyMLV cells had a very low basal level of DNA fragmentation unaffected by sustained levels of cAMP (Fig. 2, lanes 6 and 7). PC E1A cells (Fig. 2, lanes 4 and 5) and PC HE4 cells (Fig. 2, lanes 10 and 11) showed an increase in DNA fragmentation in the presence of a sustained increased cAMP level. Finally, PC E1A + PyMLV cells (Fig. 2, lanes 8 and 9) had a basal level of DNA fragmentation, which was not increased by the concomitant presence of IBMX and TSH.

Effect of IBMX on levels of intracellular cAMP stimulated by TSH in PC E1A cells

Because transformation interferes with several steps of the pathways leading to cell proliferation, we examined the adenylate cyclase in the PC E1A cell line. PC Cl 3 and PC E1A cells were treated with 1 nM TSH in the presence and in the absence of 1 mM IBMX for various lengths of time, and cAMP was measured thereafter. In both wild-type and transformed cells, TSH-stimulated intracellular cAMP levels reached a peak after about 30 min, and declined thereafter. Twelve hours after TSH stimulation, the intracellular levels of cAMP were reduced by 50% in PC Cl 3 cells and by 80% in PC E1A cells (Fig. 3). IBMX treatment prevented the decrease in cAMP intracellular levels (Fig. 3). The kinetics of the TSH-dependent intracellular cAMP accumulation was almost identical in the PC Cl 3 and in the PC E1A cells, although the magnitude of the increase was 3-fold lower in the latter.

Effect of steadily elevated levels of cAMP on protein kinase activity

cAMP-dependent protein kinase A (PKA) is considered the major intracellular target of cAMP. PKA transduces the message of hormones that activates the adenylate cyclase–cAMP pathway, through the phosphorylation of as yet unknown cellular substrates (13, 15, 16). To investigate whether the inhibitory effect on mitogenesis induced by steadily elevated levels of cAMP involves PKA activity, we analyzed PKA in PC Cl 3 cells in the presence of TSH alone or in association with IBMX.

We measured PKA activity (evaluated as the ability to phosphorylate a synthetic substrate in vitro) in total extracts of cells treated with TSH and/or IBMX for different lengths of time. In PC Cl 3 cells, PKA activity reached a peak after 8 h of TSH stimulation and declined thereafter, as demonstrated by the kinetics of
the residual cAMP-inducible kinase activity in vitro. With concomitant IBMX and TSH, PKA activity peaked at about 8 h, but contrary to TSH alone, remained steadily elevated thereafter (Fig. 4). A similar effect was obtained in the transformed cells, in spite of individual variations in the kinetics of PKA in the various cell lines. PKA was TSH-activated in all the transformed cells (Fig. 4). Concomitant treatment with TSH and IBMX maintained the PKA in an activated state up to 18 h (Fig. 4). Basal Kemptide phosphorylation (without cAMP) was not significantly different in the whole extract of the various cell lines treated with TSH alone or in the presence of IBMX.

**Discussion**

Transformation of thyroid cells in culture, by means of oncogene transfection or viral infection, gave rise to cell lines that proliferated also in the absence of TSH. However, some transformed thyroid cell lines
maintained their ability to respond to TSH stimulation. Thus, since TSH exerts its biological activity via the adenylate cyclase/cAMP system, some transformed cells, similar to non-transformed cells, are still able to respond to the cAMP pathway (17, 18).

Here we have investigated how different oncogenes causing different phenotypes influence thyroid cell sensitivity to the duration of cAMP increase.

Treatment of transformed thyroid cells with TSH and with the phosphodiesterase inhibitor, IBMX, interferes with cell proliferation in a manner depending on the oncogene used for transformation and does not correlate with the degree of malignancy. In fact, in PC Cl 3 cells carrying the middle T of the polyoma virus and expressing a transformed phenotype (PC PyMLV cells), sustained increase of cAMP results in a block of cell proliferation, suggesting a cytostatic effect of sustained levels of cAMP. On the other hand, in PC Cl3 cells carrying E1A (PC E1A) and lacking the expression of a fully transformed phenotype, sustained cAMP levels induced a decrease in cell number (from 2.5 × 10^5 at the day of plating to 2.7 × 10^4 at day 7). The observation suggests, in this case, a cytotoxic effect of sustained levels of cAMP. Thus, the presence of the E1A gene induces in the thyroid cells the expression of a phenotype extremely sensitive to sustained levels of cAMP. Finally, in cells carrying both oncogenes and expressing a highly malignant phenotype, sustained levels of cAMP induce a significant decrease in cell number, although less dramatic than that induced in cells carrying E1A alone (from 2.5 × 10^5 at the day of plating to 5.9 × 10^4 at day 7). These observations suggest that PC E1A + PyMLV cells express a sort of intermediate phenotype, between that expressed by PC PyMLV and PC E1A, as if polyoma virus middle T gene can exert partial protection against the cytotoxic effect of sustained levels of cAMP.

These data confirm that in thyroid cells, both wild-type and transformed, only an acute and transient increase of cAMP intracellular levels can activate the cascade of events controlling cell proliferation; if cAMP decrease is blocked, proliferation is inhibited. Thus, although the three transformed thyroid cell lines employed proliferate in the absence of TSH and, in turn, of cAMP, they retain a molecular machinery able to discriminate between an acute and transient increase and a stable and sustained elevation of intracellular levels of cAMP.

PKA represents the major intracellular receptor of cAMP, and it is generally accepted that cAMP exerts its biological effect through PKA. The kinetics of PKA activity is biphasic in thyroid cells stimulated with TSH (13), an increase followed by a decline, as is that of TSH-stimulated cAMP levels. In the presence of steadily elevated levels of cAMP, PKA increases and remains elevated for at least 18 h in both normal and transformed cells. Although phosphorylation induced by PKA appears to be necessary for initiating the cell cycle, it is conceivable that the decline of PKA activity is also essential for the control of mitogenesis. Sustained levels of cAMP could act either by maintaining PKA continuously activated or by inhibiting the activity of a specific phosphatase. Therefore, also in transformed cells, regulation of cell proliferation is not only dependent on the concentration of second messenger, and the activation of the appropriate post-receptor pathways, but also on the timing of persistence of the various intracellular signal(s).
It is generally accepted that both E1A gene and cAMP play a role in the activation of the apoptotic process in many cell systems (19–23). Data presented here indicate that the ability of the sustained levels of cAMP to induce apoptosis in transformed thyroid cells depends upon the oncogene expressed. In fact, a sustained increase in cAMP induces apoptosis only in thyroid cells carrying the E1A gene alone (PC E1A cells). PC Cl 3 cells carrying the middle T antigen from the polyoma virus (PC PyMLV) undergo spontaneous apoptosis, but the extent of the phenomenon is very modest and persistently elevated levels of cAMP do not increase it. Finally, cooperation between E1A and middle T genes, concomitantly expressed by PC E1A+PyMLV cells, results in an increase of basal apoptosis that cannot be further augmented by sustained levels of cAMP.

These results suggest that in thyroid cells transformation by E1A induces a phenotype remarkably sensitive to steadily elevated levels of cAMP. The region of protein E1A essential for induction of apoptosis is exon 1, which is required for binding to cellular protein p300 and pRb (24). Preliminary observations from our laboratory (A Fusco & F Trapasso, unpublished observation) suggest that binding of p300 to E1A may play a critical role in regulating the sensitivity of PC Cl 3 cells transfected with the E1A oncogene to sustained versus transient cAMP intracellular levels. It has been reported that in normal rat kidney, the presence of E1A caused apoptosis only in growth-inhibited cells and not in actively growing cells (25). The block of proliferation induced in PC E1A cells by elevated levels of cAMP could be the trigger for the apoptotic process specifically induced by E1A in transfected thyroid cells.

At present a direct role of polyoma virus middle T gene in induction or regulation of the apoptotic process is still under investigation and very few reports in the

**Figure 4** The effect of steadily elevated levels of cAMP on PKA. PKA activity was measured, in a whole extract of cells treated with TSH and/or IBMX for different periods of time, as the ability to phosphorylate Kemptide, a synthetic substrate, *in vitro*. PKA activity was monitored by incorporation of [γ-32P]ATP in the absence (■ and ●) or in the presence (X and ■) of 5 μM cAMP. In the extracts of the four cell lines tested treated with 1 nM TSH alone (●), cAMP-inducible kinase activity decreased between 6 and 10 h of TSH stimulation, depending on the cell lines, and increased thereafter. In the presence of IBMX and TSH (X), cAMP-inducible kinase activity rapidly decreased and remained stable thereafter. The values reported represent the mean ± S.D. of four independent samples for each experimental point.
literature deal with this topic. Webster et al. (26) reported that in transgenic mice the expression of a mutant of middle T gene defective in recruiting the phosphatidylinositol 3' (PI3) kinase induced a phenotype highly apoptotic in mammary epithelia. Dahal et al. (27) showed that a mutant of polyoma virus middle T gene unable to activate PI3 kinase induced apoptosis. On the other hand, Borde et al. (28) observed that the presence of polyoma virus middle T partially prevented apoptosis induced by restrictive growth conditions in a permanent glial precursor cell line immortalized with the adenovirus E1A gene.

In the thyroid system the presence of middle T gene alone induces apoptosis and, different from the data reported above, synergies with E1A in this direction: surprisingly, middle T gene expression seems to afford protection only from the E1A-dependent cAMP-induced apoptosis. Thus, the two genes coexisting in a thyroid cell work together, although through differing pathways, to induce apoptosis, but polyoma virus middle T is dominant on the E1A gene in canceling the apoptotic potential of sustained intracellular levels of cAMP. In this experimental model, sensitivity to apoptosis, induced by sustained elevated levels of cAMP, is inversely correlated with malignancy. In fact, both PC PyMLV and PC E1A+PyMLV cells, which express the more malignant phenotype, do not undergo further apoptosis in the presence of steadily elevated levels of cAMP.

The ability of normal and transformed thyroid cells to respond differently to sustained versus transient intracellular concentrations of cAMP can be a powerful tool for identifying and dissecting the various steps leading to cAMP-dependent cell growth and, in addition, the step(s) where various types of oncogenes can interfere with physiological growth pathways.

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