Effect of A549 neuroendocrine differentiation on cytotoxic immune response

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Abstract

The present study was designed to determine the effects of factors secreted by the lung adenocarcinoma cell line with the neuroendocrine phenotype, A549NED, on cytotoxic T lymphocytes (CTLs) activity in vitro. A perspective that integrates the nervous, endocrine and immune system in cancer research is essential to understand the complexity of dynamic interactions in tumours. Extensive clinical research suggests that neuroendocrine differentiation (NED) is correlated with worse patient outcomes; however, little is known regarding the effects of neuroendocrine factors on the communication between the immune system and neoplastic cells. The human lung cancer cell line A549 was induced to NED (A549NED) using cAMP-elevating agents. The A549NED cells showed changes in cell morphology, an inhibition of proliferation, an overexpression of chromogranin and a differential pattern of biogenic amine production (decreased dopamine and increased serotonin [5-HT] levels). Using co-cultures to determine the cytolytic CTLs activity on target cells, we showed that the acquisition of NED inhibits the decrease in the viability of the target cells and release of fluorescence. Additionally, the conditioned medium of A549NED and 5-HT considerably decreased the viability and proliferation of the Jurkat cells after 24 h. Thus, our study successfully generated a neuroendocrine phenotype from the A549 cell line. In co-cultures with CTLs, the pattern of secretion by A549NED impaired the proliferation and cytotoxic activity of CTLs, which might be partly explained by the increased release of 5-HT.

Introduction

Lung cancer is a leading cause of death due to cancer in both sexes worldwide (1, 2). A primary concern of this disease is the neuroendocrine phenotype linked to decreased survival and increased peripheral tumour cells, drug-resistant tumours and metastasis (3, 4). The main characteristics of tumours with a neuroendocrine phenotype are low proliferation rates, polygonal morphology, dense chromatin and a specific distribution pattern, that is nests, trabecular, acinar and rosette-like structures (5). Such tumours also express neuroendocrine markers such as chromogranin A (CgA) (6), synaptophysin, neuronal enolase and CD56 (7, 8, 9, 10). A key aspect of the neuroendocrine phenotype is the formation of granules that secrete various factors, including hormones and neurotransmitters. In the recent years, there has been an increasing interest in the role of these factors as immunomodulators in bidirectional communication with the immune system (11). In some cancers, it has been proposed that new cells with neuroendocrine-like properties originate from pre-existing epithelial cancer
cells via neuroendocrine differentiation (NED) (12). NED can be replicated in vitro by the exposure of cells to agents that elevate or mimic intracellular cAMP (i.e. 8-Br-cAMP, isobutylmethylxanthine and forskolin) (13, 14), androgen depletion (15), interleukin 6 (16), ionising radiation (17) and contact inhibition promoted by a high cellular density (12).

Although several studies have examined NED in lung cancers, the effects of secreted immunomodulators and their associations with antitumour immune responses remain unclear. This study aimed to determine the effects of factors secreted by the lung adenocarcinoma cell line A549 with NED (A549_NED) on cytotoxic T lymphocytes (CTLs) activity in vitro.

Materials and methods

Cells and reagents

Jurkat human lymphoblast cells (TIB-152) were purchased from ATCC, and the A549/GFP cell line (AKR-209) from human lung adenocarcinoma was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). DMEM, RPMI-1640, foetal bovine serum (FBS) and phytohaemagglutinin A (PHA) were purchased from Gibco. The monoclonal antibody anti-CgA was purchased from BD Biosciences. 3-isobutyl-1-methylxanthine (I7018), forskolin (F6886) and serotonin (H9523) were purchased from Sigma-Aldrich. All other chemicals were of the highest grade of purity commercially available.

Cell culture

A549/GFP cells and co-cultures were maintained in DMEM, and Jurkat cells were cultured in RPMI-1640. All media were supplemented with 10% FBS, 100 KU/L penicillin and 100 KU/L streptomycin from Gibco at 37°C with a 5% carbon dioxide-enriched air atmosphere.

Neuroendocrine differentiation induced by cAMP-elevating agents

The human lung adenocarcinoma A549 cells were seeded at a density of $2 \times 10^5$ cells/mL in 12-well plates in 1000 L triplicate aliquots in DMEM for 24 h. The wells were washed with PBS and then treated for 24, 72, 120 and 144h with 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), forskolin (FSK, 0.5 mM) or IBMX+FSK (I+F) in fresh medium. Morphologic changes and proliferation rates were evaluated via light microscopy. The conservation of morphologic changes at 24 or 48h after treatment withdrawal (WD) was also assessed. Cells were collected to analyse the expression of neuroendocrine markers and evaluate the immunomodulator effects.

Proliferation assay

The proliferation rate of NED was determined by culturing $2 \times 10^5$ A549 cells/mL in 12-well plates in 1000 L triplicate aliquots in DMEM. These cells were stained with 100 μL of 0.04% trypan blue solution to evaluate the cell viability of each treatment at 24, 48, 72, 96 and 120h.

To evaluate the direct effect of the soluble secretion factors, we used conditioned medium and different concentrations of serotonin (5-HT). The conditioned medium (CM) supplemented with the soluble secretion factors released by A549_{CTRL} and A549_{NED} was collected for 48h after the treatment for differentiation. CM or 5-HT was added to the Jurkat cells, and proliferation was determined by culturing $2.5 \times 10^5$ cells/mL in 12-well plates in 1500 L triplicate aliquots. Cells were stimulated with 0.02 mL of PHA (Gibco) for 24 h for the activation of CTLs. Naive or activated T-cells were then supplemented with 5-HT at final concentrations ranging from $1 \times 10^{-12}$ to $1 \times 10^{-3}$ M for each treatment. Finally, cells were stained with 100 μL of 0.04% trypan blue solution to evaluate the cell viability at 24 and 28h.

Flow cytometry

Flow cytometry was used to identify the neuroendocrine marker, CgA. Briefly, cells were resuspended in 500 μL PBS, and 20 μL of the anti-CgA-PE monoclonal antibody (clone S21-537, BD) was added. The mixture was incubated for 30 min at room temperature and protected from light. After incubation, the cells were washed twice with PBS and resuspended in 500 μL PBS. Samples were acquired on a FACSVersa flow cytometer (BD Biosciences). Data analysis was performed using FlowJo trial version (18).

Biogenic amine determination

Frozen pellets of cells were used to determine the levels of the neurotransmitters nonadrenaline (NA), adrenaline (AD), dopamine (DA) and 5-HT using reversed-phase chromatographic analysis (RP-HPLC). Briefly, the biogenic amines were extracted using 400 μL of an extraction buffer containing 5% ascorbic acid, 200 mM sodium phosphate, 2.5 mM L-cysteine and 2.5 mM EDTA. The protein was then
precipitated by adding 100 μL of 0.4 M perchloric acid and incubated at 20°C for 20 min. Supernatants were collected after centrifugation at 12,000 rpm for 10 min (4°C). The samples were filtered by 0.22 μm before injection. NA, AD, DA and 5-HT concentrations were determined using RP-HPLC in a system integrated with a PU-2089plus pump (Jasco, Inc., Tokyo, Japan), an AS-2057plus autosampler (Jasco) and an X-LC™3120FP fluorescence detector (Jasco). The instruments were controlled using ChromNAV software (Jasco). Chromatographic runs were performed using a Jupiter C18 column (300A, 5 μ, 4.6×250mm; Phenomenex, Torrance, CA, USA) at 30°C. The column was equilibrated with mobile phase A containing 0.1% trifluoroacetic acid. Mobile phase B (MPB) containing 0.1% trifluoroacetic acid in acetonitrile was used to perform a linear gradient until 20% MPB from min 5 to min 15; 20% MPB was then maintained until min 20. The flow rate was 0.8 mL/min. The fluorescence detector was set at gain 1000, attenuation 32, response 20s and 280 nm for excitation and 315 nm for emission. Sample injection volume was 50 μL. The order and retention times of the eluted monoamines and their metabolites in the chromatogram were NA (5.5 min), AD (7 min), DA (11.2 min) and 5-HT (16.1 min).

**In vitro cytology assay**

After inducing NED using cAMP-elevating agents, the A549 cells (target cells) with the constitutive expression of GFP were incubated with the Jurkat cells (CTLs) resting or preactivated with 20 μg/mL PHA as effector cells at 37°C for 6 or 24 h in the darkness. Culture medium without phenol red was added to the target cells to determine proliferation, cell viability and the minimum and maximum release of fluorescence. Fluorescence of supernatants was read using the fluorimeter (Varioskan) with excitation at 410 nm and emission at 520 nm.

**Receptor detection via western blot**

The expression of 5-HT5A and 5-HT7 receptors in the Jurkat cell line was determined via western blot (WB), and the total protein was extracted from the Jurkat cells following the manufacturer’s protocol.

Briefly, the Jurkat cells were solubilised for 30 min at 4°C in a lysis buffer containing 25 mM Tris-HCl, pH 7.1, with a protease inhibitor cocktail (Complete Mini, Roche). After centrifugation, the protein concentration was quantified via the Bradford method. Total proteins were denatured at 85°C for 5 min, subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline-0.05% Tween 20 PBS containing 5% non-fat dry milk for 1 h at room temperature. The blots were then incubated with polyclonal anti-human receptor antibody (5-HT5A and 5-HT7; Santa Cruz Biotechnology) for 2 h, washed in PBS and incubated overnight with a secondary antibody linked to horseradish peroxidase (Invitrogen). Finally, the bound horseradish peroxidase was visualised using a high-sensitive chemiluminescence system (ECL Kit; GE Healthcare).

**Statistical analysis**

Data are expressed as mean ± error. Differences between the experimental groups were analysed using one-way ANOVA and Tukey’s significant difference or Dunnett’s test. Differences with *P*<0.05 were considered statistically significant.

**Results**

Consistent with previous studies in which cAMP-elevating agents induced NED in NSCLC cells, we successfully induced NED in A549 (A549<sub>ned</sub>) via stimulation with IBMX, FSK and I + F (19). Morphologic changes induced by cAMP-elevating agents were observed via light microscopy at 24, 72 and 120 h. Figure 1 shows that treatment with IBMX (0.5 mM) or FSK (0.5 mM) promoted an increase in the cell size as well as the formation of new neurite-like projections after 72 h (20). Treatment with I + F promoted morphology changes after 24 h, including rounding of the cell body and extension of neurite-like cellular processes (21, 22). Lower concentrations (0.25 mM and 0.125 mM) did not promote morphologic changes (data not shown).

As shown in Fig. 2, the treatment of cells with FSK or I + F inhibited the proliferation after 48 h, whereas the treatment with IBMX inhibited the proliferation only after 72 h.

To determine if these changes were due to NED, we quantified the expression of neuroendocrine markers in the cells treated with cAMP-elevating agents. The groups treated with FSK showed a significant overexpression of CgA (Fig. 3B).

A review by Day and Salzet (23) proposed the main aspects of NED and highlighted the importance of the formation of secretory granules expressing chromogranins. Our results support this proposal and corroborate the acquisition of the neuroendocrine phenotype in A549 cells.
To assess the changes in secretion, A549_CTRL and A549_NED were compared using HPLC to identify the presence of biogenic amines (Fig. 4). The results revealed that A549_CTRL produced EP and DA but not 5-HT or NE. Interestingly, the acquisition of NED in all three groups (i.e. by IBMX, FSK and I+F treatments) changed the pattern of biogenic amine production, showing a reduction of DA (Fig. 4B) and increase of 5-HT (Fig. 4C).

Previous studies have reported the ability of some cells to revert to their original phenotype after the acquisition of NED; this occurs in two stages, namely reversible and terminal. To evaluate the cytolytic CTLs activity over the A549 cells after the acquisition of NED, we monitored whether cAMP-elevating agents induced a permanent conversion from epithelial to neuroendocrine morphology. The phenotype obtained after 72h of treatment was retained for 24h (not shown). The morphologic changes generated after 120h of treatment were retained for 48h, as shown in Fig. 5. These results suggested the generation of a terminal neuroendocrine phenotype (24, 25, 26).

To determine the cytolytic activity of the target cells, co-cultures of A549_CTRL or A549_NED were tested against CTLs (resting and preactivated with PHA Jurkat cells). Figure 6 presents the fluorescence release of the target cells constitutively expressing GFP. The quantification of the viability of the target and cytotoxic cells are separately shown in Fig. 7.

As shown in Fig. 6A, CTLs generated an increased response of fluorescence release in accordance with the effector-to-target ratios and the length of exposure. In addition, there was a significant reduction in the fluorescence release in co-cultures with A549_NED than in A549_CTRL at 24h (85.98%±6.3% A549_NED reversible vs. 83.72%±6.3% A549_NED terminal vs. 105.7%±9.6% A549_CTRL). Figure 6B presents an overview of these results.

The viability of the target cells in co-cultures was assessed to corroborate these results. The co-culture of A549_CTRL vs the Jurkat cells preactivated with PHA showed a decrease of 54.02 % of viability compared with that of the A549 cells alone (Fig. 7A). In contrast, the previous acquisition of NED in all groups inhibited the decrease of

Figure 1
Effect of cAMP-elevating agents on A549 cell morphology. A549 cells were treated for 24, 72 or 120 h with IBMX (0.5 mM), FSK (0.5 mM) or I+F. Bars on the photomicrographs correspond to 200 µm. Figures are representative of three independent experiments for triplicate. FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine.
the viability of the target cells in 6 h co-cultures (A549\textsuperscript{CTRL} 54.02% vs. A549\textsuperscript{NED} (i + f) 96.14%), as shown in Fig. 7A.

A further analysis of 6 h co-cultures showed that co-cultures with A549\textsuperscript{NED} significantly decreased the viability of the Jurkat cells preactivated with PHA (Fig. 7B).

In addition, to clearly identify whether this impairment of the viability of the Jurkat cells was due to the secretion of A549\textsuperscript{NED} cells, the 48 h CM from A549\textsuperscript{CTRL} and A549\textsuperscript{NED} was collected to posteriorly assess the influence of secretion on the viability MTT and proliferation (trypan blue) of the Jurkat cells at 24 and 48 h. The results showed that CM decreased the proliferation (Fig. 8A) and viability (Fig. 8B) of the resting Jurkat cells after 24 h (significantly, with forskolin treatments).

To evaluate the direct effect of 5-HT on the proliferation of the Jurkat cells, we explored the proliferation rate of the resting and preactivated with PHA Jurkat cells at increasing concentrations of 5-HT (from $1 \times 10^{-12}$ to $1 \times 10^{-3}$ M) for 24 and 48 h. At 24 h of treatment, there was no significant difference between the control and supplemented groups (Fig. 9A). At 48 h, as shown in Fig. 9B, all 5-HT treatments, except $1 \times 10^{-5}$ M, significantly inhibited the proliferation rate of the Jurkat cells preactivated with PHA. To confirm the expression of 5-HT receptor, we used WB to examine protein lysates from Jurkat T cells. Figure 9C shows the expression of 5-HT5A and 5-HT7 receptors in the Jurkat cell line.

**Discussion**

The present study was designed to determine the effects of the factors secreted by the lung adenocarcinoma cell line with the neuroendocrine phenotype A549\textsuperscript{NED} on CTLs (Jurkat) activity in vitro.

The origin of neuroendocrine cells in tumours and the underlying molecular mechanisms remain controversial. Nevertheless, previous studies have reported that the sustained elevation of intracellular cAMP by cAMP analogues, adenylate cyclase agonists (i.e. FSK) or phosphodiesterases (PDEs) inhibitors (i.e. IBMX) may promote the acquisition of neuroendocrine characteristics via two main mechanisms (Fig. 10), namely a classical pathway that involves the release of the catalytic subunits of PKA and an alternative pathway that promotes the activation of the EPAC pathway (13, 14, 26).
As shown in Fig. 10, the activation of PKA promotes the phosphorylation of the ser133 in CREB. This phosphorylation stimulates the translocation of bHLH transcription factors such as ND1, ASCL1 and TTF1 that upregulate the expression of functional molecules such as NCAM1 and SYP, respectively. The knockdown of the RE1-silencing transcription factor (REST) also contributes to the upregulation of SYP and expression of neuroendocrine genes with RE1 domains like CgA (13, 20, 21, 26, 27).

**Figure 4**
Effect of NED on the pattern of biogenic amine secretion. The A549 cells were exposed to cAMP-elevating agents for 120h for transdifferentiation. Biogenic amines were then quantified using HPLC in the samples A549CTRL, A549IBMX, A549FSK and A549 (NED)(i + f) and compared to the standard mixture of biogenic amines: NA, (A) AD, (B) DA and (C) 5-HT. The experiments were repeated thrice with independent samples. ND, not detectable. n=3 for triplicates. Values are expressed as mean ± error. One-way ANOVA with Tukey’s post hoc test. Bars with different letters represent statistical significance (P<0.01). DA, dopamine; AD, adrenaline; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; NED, neuroendocrine differentiation; NA, nonadrenaline; 5-HT, serotonin.

**Figure 5**
Cell morphology with neuroendocrine differentiation (NED) after withdrawal (WD) of cAMP-elevating agents. The A549 cells were exposed to cAMP-elevating agents for 120h for transdifferentiation. Cell morphology was assessed 24 and 48h after WD. Bars on the photomicrographs correspond to 200µm. Figures are representative of three independent experiments for triplicate.
The novel mechanism for NED described in PC12 and LNCaP cells states that the elevation of intracellular cAMP simultaneously promotes the binding of cAMP to a Rap1-specific guanine exchange factor, Epac1, which causes a conformational change and leads to an enhanced exchange activity towards Rap1, which is localised on intracellular membranes. The GTP-bound Rap1 then stimulates the effector B-Raf to activate the MAP kinases, MEK and Erk1/2, to upregulate the transcription of two genes, namely Egr1 and Vilin2, which promote neurite growth and increase the cell size, as shown in Fig. 10 (27, 28, 29).

Here, we observed morphological changes in the treated groups after 72 h, in accordance with previous results obtained in NCI-H157 and Lu-CSF1 lung cancer cell lines (13), which might be attributed to the upregulation of Egr1 and Vilin2 (16). Combined treatments produced changes more rapidly than did the individual drugs, suggesting an additive effect due to the mechanism of the elevation of intracellular cAMP. FSK is more specific to activating adenylate cyclase, whereas IBMX may promote a crosstalk via the inhibition of phosphodiesterase. Although cAMP and cGMP are synthesised by different enzymes, they are both degraded by the large superfamily of PDEs, promoting the posterior stimulus of cAMP and cGMP concentration and the posterior activation of PKG (30, 31).

Jones & Palmer have previously demonstrated the ability of FSK to induce early changes in the cell morphology via the phosphorylation of RhoA, stimulating actin polymerisation (21); thus, combined treatments may favour this mechanism via the activation of PKG. Both protein kinases have been reported to phosphorylate active, membrane-bound RhoA and promote its translocation to the cytosol, impairing the activation of its effector Rho kinase, which is well known for its role in the modification of the actin cytoskeleton (32).
The results of this study suggest the generation of a terminal neuroendocrine phenotype after 120 h of treatment. Our finding of a terminal phenotype is consistent with the results of Wang et al. who observed a non-reversible phenotype in the lung cells for 14 days after 120 h of treatment with a mixture of KGF, IBMX, 8-Br-cAMP and dexamethasone (24, 26, 33). Our finding of a decreased proliferation rate corresponds with the findings of Cox et al. (26, 34) who reported a similar inhibition of thymidine incorporation after the exposure of cells to cAMP-elevating agents (dbcAMP, IBMX and FSK) in prostate adenocarcinoma cell lines (LNCaP and C4-2). According to the findings of Pernicová et al. (12) obtained in prostate adenocarcinoma cell lines (LNCaP and LAPC-4), the decreased proliferation rate might be due to more cells in arrest (G0 phase) as a result of NED. The stimulation of Epac2 in bronchoalveolar cells (35) is associated with cell cycle arrest by the phosphorylation of p38 (36) as well as with apoptosis by the activation of Akt (37). Further evidence is necessary to understand the role of these pathways in our results.

The overexpression of the neuroendocrine marker CgA in our study was consistent with the results reported by Chang et al. in the A549 cell line (14) and those reported by Pernicová et al. in LNCaP cells (12). In lung cancers, it has been demonstrated that REST1 is highly expressed in NSCLC cells but transcriptionally repressed in SCLC cells. The inactivation of REST1 via methylation is directly related to the expression of the neuroendocrine biomarkers, synaptophysin and CgA (4). According to Day & Salzet (23), the expression of chromogranin does not imply that the cell has a neuroendocrine origin but that it has acquired a neuroendocrine phenotype. In this sense, our results provide a strong evidence of NED of the A549 cell line. The acquisition of neuroendocrine characteristics could be the result of a genetic switch that induces the expression or inhibits repressors that prevent the inhibition of the neuroendocrine markers (23).

According to Cerasuolo (2015), the ability of neuroendocrine cells to induce an ‘early onset of a
hormone-refractory status’ is intriguing and clinically relevant (20). Therefore, the data of the differential pattern of neurotransmitter production support the idea that peptide hormones or biogenic amines can either be released into the bloodstream or can locally act by promoting paracrine interaction with the tumour microenvironment, generating worse prognostic outcomes for patients. Our observations of A549

Figure 10
Proposed mechanism of neuroendocrine differentiation by cAMP-elevating agents. Solid line indicates the reported pathways in the lungs, and the dashed line indicates the pathways reported in other tissues. ASCL1, achaete-scute homolog like 1; CgA, chromogranin A; Epac1 and Epac2, guanine exchange factor; Egr1, early growth response protein 1; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; ND1, NeuroD1; NCAM1, neural cell adhesion molecule 1; PDE, phosphodiesterase; PKA, protein kinase A; REST1, repressor element 1-silencing transcription factor; Rap1, small GTPase Ras-related protein; SYP, synaptophysin; (serine/threonine-protein kinase) B-Raf, MEK, ERK1/2 and p38, mitogen activated protein kinases; VI2, Vilin2.

Co-cultures of cytotoxic vs target cells were used to obtain information on the immunomodulatory effects of the soluble factors produced by the A549

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possibly through the reduced expression and distribution of IL-2 receptors (47, 48).

Thus, our results suggest that altered biogenic amine production in A549\textsubscript{NED} can be used as a defence mechanism against CTLs activity (49).

**Conclusion**

In conclusion, the present study reported the following findings:

1. **The generation of a neuroendocrine phenotype from the A549 cell line (A549\textsubscript{NED}) that was stable for 48h after stimuli WD and expressed neuroendocrine protein markers.**

2. **The transdifferentiated human lung adenocarcinoma cell line (A549\textsubscript{NED}) acquired a differential pattern of biogenic amine production compared with the control cells, specifically producing less DA and more 5-HT.**

3. **In co-cultures with the Jurkat cells as CTLs, the acquisition of neuroendocrine characteristics correlated with diminished cytolysis.**

4. **CM of the transdifferentiated human lung adenocarcinoma cell line (A549\textsubscript{NED}) independently impaired the viability and proliferation of CTLs.**

5. **5-HT alone impaired the proliferation of the PHA-activated CTLs.**

**Declaration of interest**

The authors declare that they have no competing interests. I M performed neuroendocrine transdifferentiation, co-cultures and prepared the manuscript. N A R E participated in the design of the study, analysed the results and performed the statistical analysis. A R C corroborated the effect of 5-HT in the Jurkat cells and determined the presence of receptors. G P S and L P analysed and determined the biogenic amines using HPLC and analysed the results. G G A reviewed the manuscript. L C B provided coordination and helped to design the study. All authors read and approved the final manuscript.

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