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SLURP-1 and -2 in normal, immortalized and malignant oral keratinocytes

Juan Arredondo, Alexander I. Chernyavsky, and Sergei A. Grando

Department of Dermatology, University of California, Davis, CA, USA

Abstract

The secreted mammalian Ly-6/urokinase plasminogen activator receptor-related proteins (SLURP)-1 and -2 are produced by keratinocytes comprising the mucocutaneous epithelium. They regulate in autocrine and paracrine ways cell growth and differentiation through the nicotinic acetylcholine receptors (nAChRs) expressed on the plasma membrane. Keratinocyte nAChRs are targeted by tobacco-derived carcinogenic nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) that can induce tumorigenic transformation of Het-1A keratinocytes. In this study we asked if SLURPs could abolish tumorigenic effects of nitrosamines. Preincubation with either recombinant SLURP-1 or -2 in both cases considerably reduced the number of colonies in soft agar, and the number of tumor nodules >0.5 cm in diameter in Nu/Nu mice produced by Het-1A cells treated with nitrosamines. The levels of SLURP-1 and -2 mRNA transcripts in nitrosamine-transformed Het-1A cells as well as in the tumor cell lines SCC-25 and FaDu were significantly ($p < 0.05$) less compared to normal gingival keratinocytes, which are probably the major source of the secreted SLURPs found in a sample of human saliva. The expression of SLURPs was decreased due to gene silencing of different nAChR α subunits with small hairpin RNA, suggesting that a positive feedback regulation is altered in malignant cells. Thus, SLURP-1 and -2 are efficient autocrine and paracrine ligands of keratinocyte nAChRs capable of preventing tobacco nitrosamine-induced malignant transformation of oral cells. These "proof-of-concept" preliminary results have salient clinical implications.

Keywords

keratinocytes; nicotinic acetylcholine receptors; SLURP-1; SLURP-2; nitrosamines; head and neck cancer

INTRODUCTION

Recent research has indicated that novel proteins termed SLURP (secreted mammalian Ly-6/urokinase plasminogen activator receptor-related protein)-1 and -2 act as cholinergic signaling molecules in the human mucocutaneous epithelia (Arredondo et al., 2006c; Arredondo et al., 2005b; Chimienti et al., 2003; Tsuji et al., 2003). We cloned both human SLURPs, produced recombinant proteins, rSLURP-1 and -2, and generated mouse monoclonal antibodies that visualized SLURP-1 and -2 in the cytoplasm of normal human epidermal and oral

Correspondence to: Sergei A. Grando, MD, PhD, DSc, Department of Dermatology, University of California Davis Medical Center, 3301 C Street, Suite #1400, Sacramento, California 95816, USA. Phone (916) 734-6057; Fax: (916) 442-5702; E-mail: sagrando@ucdavis.edu

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keratinocytes. Both SLURPs were found in culture supernatants of normal human keratinocytes and serum. Radioligand binding inhibition studies showed that *r*SLURP-1 ligated the conventional ligand binding site of keratinocyte nicotinic acetylcholine receptors (nAChRs), showing a higher affinity to the [³H]nicotine-, compared to the [³H]epibatidine-sensitive nAChRs. In contrast, *r*SLURP-2 showed a higher affinity to the [³H]epibatidine- compared to the [³H]nicotine-labeled sites. Experimental results also demonstrated that *r*SLURP-1 increases the activities of caspases 3 and 8, and the number of TUNEL positive cells. In contrast, *r*SLURP-2 upregulated growth of keratinocytes and their resistance to apoptosis. The differences in effects of SLURP-1 and -2 can be explained by their differential binding to keratinocyte nAChR subtypes. These findings revealed a novel paradigm of the physiologic regulation of the mucocutaneous epithelial cells by locally produced small hormone-like peptide molecules acting *via* keratinocyte nAChRs, and opened novel direction toward better understanding and treating the oral disease.

The keratinocyte nAChRs have been shown to mediate pathobiologic effects of the tobacco and nicotine derivatives on non-neuronal cells (reviewed in (Minna, 2003)). Recently, it has been demonstrated that nAChRs play an important role in mediating malignant cell transformation of epithelial cells by the tobacco-derived carcinogenic nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) (Arredondo et al., 2006a; Arredondo et al., 2006b). To elucidate signaling mechanisms, we studied transcription of the genes encoding the cell cycle, apoptosis and signal transduction regulators. The nitrosamine treated Het-1A cells showed multifold increases of the PCNA and Bcl-2 mRNAs as well as an upregulated expression of the transcription factors GATA-3, NF- κ B, and STAT-1, which could be abolished in the presence of nicotinic antagonists (Arredondo et al., 2006b). These results supported novel concept of receptor-mediated action of NNK and NNN, placing cellular nAChRs in the center of the pathophysiologic loop, and suggested that nAChR ligands may serve as therapeutic and/or chemopreventive agents.

In this study, we obtained preliminary evidence that SLURP-1 and -2 can protect cells from tumorigenic transformation. We also demonstrated that malignant transformation is associated with a decrease of SLURP production, which is regulated, in part, through cellular nAChRs.

MATERIALS AND METHODS

Cells and Reagents

Normal human gingival keratinocytes were isolated and cultured as described elsewhere (Nguyen et al., 2000). Het-1A, SCC-25 and FaDu cell lines were purchased from ATCC (Manassas, VA). Both *r*SLURP-1 and -2 and monoclonal antibodies to each SLURP protein were produced as previously described (Arredondo et al., 2006c; Arredondo et al., 2005b). The anti-SLURP-1 monoclonal antibody 336H12-1A3 and the anti-SLURP-2 monoclonal antibody 341F10-1F12 are commercially available from Research and Diagnostic Antibodies (Las Vegas, NV). NNK was purchased from Toronto Research Chemicals (North York, ON, Canada) and NNN from EaglePicher Technologies (Lenexa, KS).

Nitrosamine Exposure Experiments

Approximately 80% confluent monolayers of Het-1A cells were preincubated for 16 h with 2 μ g/ml of *r*SLURP-1 or -2 and then exposed without washing to 1 μ M of NNK or NNN for 24 h. The positive controls received nitrosamines without SLURPs and negative controls were left intact. After 3 subsequent passages, the tumorigenic activities of experimental and control Het-1A cells were evaluated by plating the cells in soft agar, and injecting them subcutaneously at a concentration of 1.5×10^7 cells per each of 2 different sites on each body side of Nu/Nu mice (The Jackson Laboratory, Bar Harbor, ME). After 4 wks of growth in soft agar, the

colonies containing >50 cells were calculated. The percentage of tumor nodules >0.5 cm in diameter at the injection site in the mouse skin was computed 6 wks post-injection, as follows: 1 nodule = 25%, 2 = 50%, 3 = 75% and 4 =100%, as per published protocols (Arredondo et al., 2006b).

Immunoblotting

For immunoblotting experiments, samples of human saliva and concentrated supernatants collected over 10 days from the monolayers of normal human gingival keratinocytes or Het-1A cells were dissolved in a sample buffer, separated *via* 4-10% SDS-PAGE, and electroblotted onto a 0.2 μ m nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were developed using the ECL + Plus chemiluminescent detection system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and scanned with StormTM/FluorImager (Molecular Dynamics, Mountain View, CA).

Real-time Quantitative Polymerases Chain Reaction (qPCR) Experiments

Total RNA was extracted from Het-1A cells at the end of exposure experiments with the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in the qPCR experiments, as detailed elsewhere (Arredondo et al., 2005a). Primers for the gene encoding human SLURP-1 were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA) and the service Assays-on-Demand provided by Applied Biosystems. For SLURP-2 the following sequences were used 5'-GAGGGACTCCACCCACTGTGT-3 and 5'-GCAGCCTATGTGGCACATCTT-3 for forward and reverse primers, respectively, and 5'-CGGGTCCTCAGCAACACCGAGGAT-3 for MGB probe (Tsuji et al., 2003). The qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance to the manufacturer's protocol, as described by us in detail elsewhere (Arredondo et al., 2006c). To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data from triplicate samples were analyzed with a sequence detector software (Applied Biosystems) and expressed as mean $\pm$ standard deviation of mRNA in question relative to that of control.

Transfecting Het-1A Cells with small hairpin RNA (shRNA) Vectors

The pre-designed SureSilencingTM shRNA plasmids specific for human $\alpha 3$, $\alpha 5$, $\alpha 7$, and $\alpha 9$ nAChR subunit genes were purchased from SuperArray Bioscience Corp. (Frederick, MD). The cells were transfected with shRNAs following the standard protocol described in detail elsewhere (Lu et al., 2004). Briefly, Het-1A cells were seeded at a density of 5×10^4 cells per well of a 24-well plate, and incubated to achieve approximately 70% confluence. To each well, increasing concentrations of shRNA in the transfection solution with the TransIT-TKO transfection reagent (Mirus, Madison, WI) were added, and the transfection was continued for 16 h at 37°C in a humid, 5% CO₂ incubator. On the next day, the transfection medium was replaced by growth medium (Cambrex Bio Sciences, Walkersville, MD), and the cells were incubated for 72 h to achieve maximum inhibition of the receptor protein expression, as was experimentally determined by qPCR (data not shown).

Statistical Analysis

All experiments were performed in triplicates and the results were expressed as mean $\pm$ standard deviation. Statistical significance was determined using Student's *t*-test. Differences were deemed significant if the calculated *p* value was <0.05.

RESULTS

SLURPs Abolish Tumorigenic Effects of Nitrosamines

Previous studies revealed that the SV40 T-antigen immortalized human esophageal keratinocytes, Het-1A cells, express $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\beta 2$, and $\beta 4$ nAChR subunits (Arredondo et al., 2006b). Treatment of Het-1A cells with either NNK or NNN facilitated their anchorage independent growth and the ability to produce tumors in nude mice, both of which could be inhibited by antagonists. In this study, preincubation of Het-1A cells with either *r*SLURP-1 or -2 in both cases considerably, albeit incompletely, reduced the number of colonies in soft agar, and the number of tumor nodules >0.5 cm in diameter in Nu/Nu mice (Fig. 1). *r*SLURP-1 antagonized the tumorigenic effect of NNK more efficiently than that of NNN, whereas the anti-nitrosamine selectivity of *r*SLURP-2 was just as opposite. Bovine serum albumin, 2 μ g/ml, did not affect tumorigenic activities of NNK and NNN (data not shown), which effectively ruled out a possibility of non-specific action of SLURPs.

Decreased Production of SLURPs in Malignant Keratinocytes

Having found that SLURP-1 and -2 can abolish tumorigenic effects of nitrosamines, we hypothesized that these cholinergic peptides may protect normal keratinocytes from malignant transformation. As a first step toward elucidation of this intriguing hypothesis, we compared the levels of gene expression of SLURP-1 and -2 in normal and malignant keratinocytes. The results of qPCR demonstrated that both lines of malignant keratinocytes SCC-25 and FaDu expressed significantly ($p < 0.05$) less mRNAs encoding SLURP-1 and -2 (Fig. 2A). The Het-1A cells also contained the lowered SLURP-1 and -2 mRNA levels ($p < 0.05$) that decreased further after treatment of cells with NNK and NNN (Fig. 2A). We could not detect neither SLURP protein in the 120-fold concentrated supernatants from Het-1A cell monolayers, whereas the protein bands corresponding to SLURP-1 and -2 could be visualized in 80-fold concentrated supernatants of normal gingival keratinocytes, and in non-concentrated samples of human saliva (Fig. 2B).

Regulation of SLURP-1 and -2 Gene Expression through Keratinocyte nAChRs

The expression and function of keratinocyte nAChRs are a subject of ligand autoregulation (Arredondo et al., 2005a; Arredondo et al., 2001). Since SLURPs function as autocrine and paracrine ligands of keratinocyte nAChRs (Arredondo et al., 2006c; Arredondo et al., 2005b), we asked how functional inactivation of a single nAChR subtype would affect SLURP gene expression. In these experiments, stable silencing of the nAChR subunits $\alpha 3$, $\alpha 5$, $\alpha 7$ or $\alpha 9$ was achieved due to transfection with shRNA, and the SLURP-1 and -2 gene expression was measured by qPCR. Functional inactivation of each receptor decreased the levels of SLURP-1 and -2 gene expression (Fig. 1C). SLURP-2 was found to be more sensitive than SLURP-1. Among the experimental conditions tested, shRNA- $\alpha 9$ was most efficient in decreasing the SLURP-2 gene expression by approximately 50%.

DISCUSSION

A discovery of novel cholinergic peptides exhibiting profound biologic effects on keratinocytes, due to binding with a high affinity to cellular nAChRs, suggested that SLURP-1 and -2 might interfere with binding of the nicotine-derived nitrosamines NNK and NNN to these cells and thus protect them from tumorigenic transformation. The results of this study provide proof-of-concept results that the tumorigenic effects of NNK and NNN can be blocked by *r*SLURP-1 and -2, which has salient clinical implications. Since the effects of both nitrosamines and SLURPs are mediated by nAChRs, *r*SLURP-1 and -2 most likely exhibited their anti-tumor activity by displacing NNK and NNN from nAChRs. Lack of complete inhibition of the tumorigenic activities of nitrosamines may be due to inability to completely

block the binding of each nitrosamine by a single SLURP protein at the concentration tested. This may be addressed by increasing the concentrations of SLURPs and/combining both SLURP proteins.

The obtained results also established contribution of nAChRs to autoregulation of SLURP gene expression. Functional inactivation of specific nAChR subtypes, especially $\alpha 9$ -made channels, decreased expression of SLURP-1 and -2, indicating that nAChR signaling naturally upregulates SLURPs. Since malignant transformation of keratinocytes was found to be associated with decreased SLURP production in keratinocytes, it will be important to compare the nAChR expression in normal and malignant keratinocytes, and establish contribution of specific nAChR subtypes to mediating the oncogenic action of each nitrosamine. Perhaps, one of the physiological roles of SLURPs is protection of normal keratinocytes from malignant transformation, and a loss of this function is associated (or leads to) cancer. Future studies should gain a mechanistic insight into the nAChR-mediated actions of nitrosamines and SLURPs.

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Fig 1. Effects of *rSLURP-1* and *rSLURP-2* on the Tumorigenic Activities of NNK and NNN.

The Het-1A cells were preincubated for 16 h with 2 $\mu\text{g/ml}$ of either *rSLURP-1* or *rSLURP-2* and then exposed to 1 μM of NNK or NNN for 24 h, followed by 3 passages in culture. After that, the tumorigenic activities of experimental and control, unexposed cells were measured *in vitro* and *in vivo* as detailed in Materials and Methods. Asterisks indicate significant ($p < 0.05$) differences compared to the effects of each nitrosamine given alone.

- A.** *rSLURP-1* and -2 reduce numbers of colonies in soft agar produced by nitrosamine-treated Het-1A cells.
- B.** *rSLURP-1* and 2 reduce number of tumors nodules induced by nitrosamine-treated Het-1A cells in the skin of Nu/Nu mice.

Fig 2. Alterations of the SLURP-1 and -2 Gene Expression.

- A.** The qPCR analysis was used to detect alterations in the gene expression at the mRNA level in the malignant keratinocyte cell lines SCC-25 and FaDu, and in intact (1) and NNK (2)- or NNN (3)-transformed Het-1A cells. The results were normalized relative to the rates of expression of corresponding genes in the samples of normal human oral keratinocytes (OKC), taken as 1. All experimental samples contained significantly ($p < 0.05$) less mRNA transcripts compared to OKC. Asterisks indicate changes that significantly ($p < 0.05$) differ in nitrosamine-treated Het-1A from intact Het-1A cells.
- B.** Visualization of the 9 kDa native SLURP-1 and -2 proteins in culture supernatants of intact Het-1A cells (lane 1) and normal human gingival keratinocytes (lane 2), and in a sample of normal human saliva (lane 3) using the mouse monoclonal antibodies 336H12-1A3 and 341F10-1F12, respectively, diluted 1:100.

- C. Intact Het-1A cells were transfected with normal control shRNA (**shRNA-NC**) or shRNA directed to the specific sequences present in the nAChR $\alpha 3$, $\alpha 5$, $\alpha 7$ or $\alpha 9$ subunits, and used for quantitative analysis of the SLURP-1 and -2 gene expression by qPCR, as described in Materials and Methods.