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μ3 Opiate receptor expression in lung and lung carcinoma: ligand binding and coupling to nitric oxide release

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Abstract

The μ3 opiate receptor subtype is expressed in human surgical specimens of both normal lung and non-small-cell lung carcinoma. Nitric oxide (NO) release is mediated through the μ3 receptor, and in lung carcinoma, morphine-stimulated NO release is significantly higher and prolonged than in normal lung. Using reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis we show that specific μ opioid receptor transcripts are present in lung carcinoma and other cells with the μ3 profile. Our findings identify a unique role for the μ3 opiate receptor in opiate-mediated NO release and suggest that endogenous opiates, through their release of NO, may play a role in cancer progression.

Keywords: Opioid receptor transcript; μ Neoplasia; Nitric oxide; Morphine; Cancer; Alternative splicing; Lung

1. Introduction

To date, experiments examining the role of nitric oxide (NO) in cancer initiation and progression show that NO plays a complex role. Firstly, NO can be involved in initiating disease by damaging DNA [1]. Secondly, NO can also have a protective role: it is essential for the tumoricidal activity of immune cells [2]. Finally, NO has been shown to have tumor-promoting effects [3]. These seemingly paradoxical observations have lead to the hypothesis that NO may differentially affect tumor progression depending on the levels and timing of release [4].

Opiate receptors may also play a role in inhibiting cancer progression. In vitro, opioid agonists such as morphine inhibit growth of lung cancer cells and in vivo, the opiate antagonist naltrexone can cause regression of chemically-induced mammary tumors [5,6]. In addition, the δ opiate receptor was recently cloned from lung cancer cells [7]. In this study, we

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identify an important link between a specific μ opiate receptor subtype, μ3, and NO release in tumor cells.

Recently, we characterized a novel opiate receptor, μ3, that is opiate peptide-insensitive and opiate alkaloid-selective. We further showed that opiate alkaloids, but not opioid peptides, release NO through the μ3 receptor [8–10]. Thus, here we document the fact that opiate compounds can have paradoxical cancer outcomes since their action may depend on NO. In the present study we examined expression of the μ3 opiate receptor and opiate-mediated NO release in normal and malignant tissue.

2. Materials and methods

2.1. Isolation of human granulocytes and tissues

Blood was obtained from Long Island Blood Services (Melville, NY). Granulocytes were isolated from blood by standard Ficoll–Hypaque separation and the cells resuspended in RPMI medium. Tissue specimens (tumor and adjacent normal lung, and thyroid) were obtained from patients undergoing surgical treatment and free from therapy. Tissue and cell samples were stored in guanidine-isothiocyanate solution (4 M guanidine isothiocyanate, with 25mM sodium citrate, 0.5% sarcosyl and 0.1M β-mercaptoethanol at −70°C) until RNA extraction. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method as described elsewhere [11].

2.2. Pathological examination

Tissue obtained from resections was either frozen immediately in liquid nitrogen, embedded in optimal cutting temperature and frozen at −70°C, or formalin-fixed and paraffin-embedded. Frozen tissue was fixed before staining with 1% paraformaldehyde and stained with hematoxylin and eosin. Lung tissue pathological diagnosis confirmed the presence of poorly differentiated, non-small-cell carcinoma with squamoid and clear cell features. Lung not involved with tumor showed edema, other reactive changes and mild, irregular fibrosis. Lymphocyte infiltration of the tumor was less than 5%. Thyroid tissue showed follicular hyperplasia and random irregular scarring.

2.3. Reverse-transcriptase-polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized from 10 μg of total RNA using an oligo dT primer and 20 units of Avian Myeloblastosis Virus reverse transcriptase (First strand cDNA synthesis kit for RT-PCR, Boehringer Mannheim) in a reaction volume of 20 μl using Taq Polymerase (Perkin-Elmer) under standard conditions. The reaction was run for 35 cycles of 1 min 30 s at 94°C, 1 min at 56°C and 2 min at 72°C. RT-PCR was performed five to ten times for each sample, using different RNA preparations. Negative controls for PCR contained the reaction mixture without template. Samples were normalized by the amplification of β-actin. The outer pair of oligonucleotide primers were designed to amplify a 1435 base pair segment, from map position 17 to 1452 based on the published 5′ and 3′ untranslated region (C1, 5′-GCAGAGGAGAATGTCAGATG-3′ and C2, 5′-CTAAGCTTGGTGAAGGTCGG-3′) [12]. Oligonucleotides were synthesized in an Applied Biosystems 394 DNA/RNA Synthesizer.

2.4. Southern blotting

PCR products were separated by gel electrophoresis in 2% agarose and blotted onto nylon. They were then visualized with a digoxigenin non-radioactive nucleic acid labeling and detection system according to the manufacturer's directions (DIG DNA Labeling Kit and DIG Luminescent Detection Kit for Nucleic Acid, Boehringer Mannheim). A μ-specific probe was generated with the following PCR primers that amplify a 440 bp fragment within a single exon of the μ gene from map position 896 to 1336 [13]: primer M1, 5′-GGTACTGGGAAAACCTCGTGAA-GATCTGTG-3′; primer M2, 5′-GGTCTCTAG-TGTTCTGACGAATTCGAGTGG-3′.

2.5. Opiate binding analysis.

Binding was studied in human granulocytes, lung and non-small-cell lung carcinoma tissue. The separated materials were washed, homogenized in 50 volumes of 0.32 M sucrose (pH 7.4) at 4°C. Membrane suspensions were prepared as described in detail elsewhere [8,14,15]. Displacement analysis was carried out as indicated in the legend to Table 1

Table 1
Displacement of [^{3}H]dihydromorphine (DHM) (40 nM) by opioid and non-opioid ligands in human granulocytes, normal lung and non-small-cell lung carcinoma tissue membrane suspensions[a]

Ligand	IC_{50} (nM)		
	Granulocytes	Lung carcinoma	Normal lung
Agonists			
δ-Agonists			
DAMA[b]	> 1000	> 1000	> 1000
Deltorphin	> 1000	> 1000	> 1000
Met-enkephalin	> 1000	> 1000	> 1000
DADLE[b]	1000	> 1000	> 1000
DPDPE[b]	> 1000	> 1000	> 1000
μ-Agonists			
DAMGO[b]	> 1000	> 1000	> 1000
Dihydromorphine	33 ± 3.9	27 ± 4.2	28.5 ± 3.3
Methadone	121 ± 12	135 ± 11.3	
Morphine	29 ± 4.5	29 ± 3.7	28.3 ± 3.1
κ-Agonists			
Dynorphin 1-17	> 1000	> 1000	> 1000
Antagonists			
Naltrexone	30 ± 5.1	31 ± 3.8	33.4 ± 4.1
Naloxone	42 ± 4.7	40 ± 4.1	39.7 ± 3.8

[a] Aliquots of membrane suspensions from human granulocytes, lung non-small-cell carcinoma and normal lung (less than 9% lymphocyte infiltrate) were incubated with non-radioactive compounds at six concentrations for 10 min at 22°C and then with [^{3}H]DHM for 60 min at 4°C. One hundred per cent binding is defined as bound [^{3}H]DHM in the presence of 10 μM dextrorphan minus bound [^{3}H]DHM in the presence of 10 μM levorphanol. Thyroid tissue, which is negative for the μ3 splice variant (Fig. 2), did not exhibit a μ3 displacement profile (data not shown). IC_{50} is defined as the concentration of drug which elicits half-maximal inhibition of specific binding. The mean and SEM for three experiments is given. The displacement analysis data indicate the potency of various opioid extracts in displacing DHM and may give specific information on different receptor populations. Incubation medium for Met-enkephalin contained phosphoramidon (100 μM) and bestatin (100 μM) to inhibit enzyme action.

[b] DAMA, (D-Ala^2, Met^5)-enkephalinamide; DADLE, (D-Ala^2, Leu^5)-enkephalin; DPDPE, (D-Pen^2, D-Pen^5)-enkephalin; DAMGO, Tyr-D-Ala^2, Gly-N-Me-Phe^4, Gly(ol)^5)-enkephalin.

and as noted elsewhere [8,14,15]. Opiate antagonists/agonists were purchased from Sigma.

2.6. Direct measurement of NO release

NO released from the tissues was measured directly using a NO-specific amperometric probe (World Precision Instruments, Sarasota, FL), as previously described [15,16,17]. The system was calibrated daily using different concentrations of the nitrosothiol donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (Sigma, St. Louis, MO) to generate a standard curve. The concentration of NO gas in solution was measured in real time with the DUO 18 computer data acquisition system (World Precision Instruments). Each experiment was simultaneously performed with a control (vehicle alone) to exclude experimental drift in NO release unrelated to the study drugs. It should be emphasized that the morphine-stimulated release of NO from endothelia has been demonstrated to be inhibited by naloxone and the nitric oxide synthase (NOS) inhibitors, *N*-nitro-L-arginine and *N*-omega-nitro-L-arginine methyl ester (L-NAME) in diverse tissues [9,15–17].

2.7. Statistical analysis.

Data were evaluated using the Student's *t*-test then Sigma-Plot and -Stat (Jandel, San Rafael, CA) for

graphic representation and evaluation. Data gatherers were unaware of the experimental treatments.

3. Results

3.1. Functional expression of the μ3 opiate receptor in lung and lung carcinoma

In vivo and in vitro evidence suggests that opiate receptors may play an important role in tumor biology. To determine what type of opiate receptors are expressed in surgical specimens of normal human lung and non-small-cell lung carcinoma, we examined the displacement of ^{3}H-dihydromorphine binding with δ, μ and κ ligands (Table 1). Like the previously described μ3 receptor expressed in granulocytes and monocytes [8–10], the receptor expressed in normal lung and lung carcinoma had no affinity for any of the opioid peptides or analogues, but had high affinity for opiate alkaloids. Thyroid tissue was negative for opiate binding (data not shown). These results suggest that both normal lung and lung carcinoma express the same receptor that is present in granulocytes, the μ3-type opiate receptor.

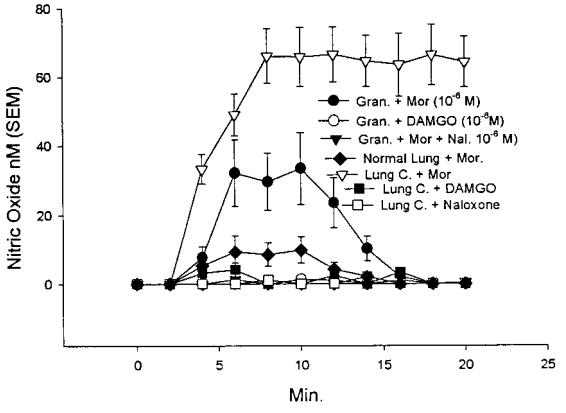

Fig. 1. Graphic representation of NO release by granulocytes (Gran.; 10^{7}/ml), normal lung and non-small-cell lung carcinoma (1 mg wet weight, including normal lung tissue) following morphine (Mor; 1 μM) exposure. Each experiment was repeated four times and performed with its own control (vehicle minus drug; NO control values for the entire experimental period was 0.2 ± 1nM). $P < 0.01$ (Student's *t*-test) comparing NO peak production between human granulocytes and non-small-cell lung carcinoma tissue at peak level times 4–12 min post exposure. Nal, naloxone; DAMGO, Tyr–Ala^{2},Gly-N-Me-Phe^{4},Gly(ol)^{5})-enkephalin.

3.2. μ3 Coupling to NO release in lung carcinoma

Previous studies have demonstrated that opiate alkaloids, but not opioid peptides, release NO through the μ3 receptor [9,15,17]. In the present study, evidence for μ3–NO coupling was obtained by direct measurement of NO levels using a NO-specific amperometric probe following stimulation with morphine (Fig. 1). Human granulocytes released NO in response to morphine (closed circles); the release was rapid and sustained, for 10–15 min; pretreatment with naloxone (closed triangles) blocked this effect. The μ opioid peptide agonist Tyr-D-Ala^{2}, Gly-N-Me-Phe^{4}, Gly(ol)^{5})-enkephalin (DAMGO) (open circles) had no effect on NO release. NO release from lung carcinoma was strikingly different (open triangles); release was more rapid, sustained for a greater period of time (>20 min) and reached peak levels of NO significantly greater (35 nM vs. 60 nM; $P < 0.005$) than that observed in granulocytes, indicating its cNOS origin. As with the granulocytes, the μ opioid peptide agonist DAMGO had no effect on NO release from lung carcinoma (closed squares). In contrast with lung carcinoma, normal lung (closed diamonds) showed little NO release in response to morphine. L-NAME (100mM) reduced the morphine-stimulated NO release from the lung carcinoma by 94% (data not shown). Thyroid tissue did not respond to morphine (10^{-6} M; data not shown). These results show that the μ3 receptor subtype identified above with detailed binding analysis (Table 1) is coupled to NO release in granulocytes, lung and lung carcinoma and that NO levels are increased by more than 6-fold in lung carcinoma compared with normal lung.

3.3. Expression of the μ receptor gene in non-neural human tissues

The results presented above show that granulocytes, lung and lung carcinoma express an opiate receptor with the μ3 phenotype. Our results further show that activation of this receptor subtype is coupled to NO release in all three tissues and that morphine-induced NO production is greatly increased in lung carcinoma. To examine the expression of μ-opiate receptor transcripts in these tissues, nested RT-PCR and Southern blot analysis were used to detect μ-specific mRNA. PCR primers derived from the 5′ and 3′ untranslated region of the μ gene [12] were used to amplify the entire predicted coding region. Using

these primers, a transcript of about 1.4 kb was seen in SH-SY5Y neuroblastoma cells (Fig. 2A, lane 1), whereas a larger approx. 2.0 kb transcript was seen in granulocytes (lane 2), non-small-cell lung carcinoma (lane 4) and normal lung (lane 5). No amplified product was obtained with thyroid tissue (lane 3).

To further characterize the large (approx. 2 kb) RT-PCR products derived from the granulocyte and lung samples, Southern hybridization of the PCR products was performed, using a 440 bp fragment derived from a single exon of the μ gene as a probe (see Section 2.4) (Fig. 1B). A positive hybridization signal was seen for the ~1.4 kb PCR product from SH-SY5Y cells (lane 1), as well as from the ~2.0 kb RT-PCR product from granulocytes (lane 2). Interestingly, a positive hybridization signal was also seen for the ~2.0 kb transcript from lung carcinoma (lane 4) and normal lung (lane 5). It was not found in samples of thyroid (lane 3). These results suggest that the μ gene may undergo alternative splicing to generate both the μ receptor expressed in SH-SY5Y cells and the novel μ3 opiate receptor subtype expressed in granulocytes, lung and lung carcinoma [18]. Interestingly, the presence of the larger 2.0 kb transcript in granulocytes, normal lung and lung carcinoma correlates with our functional studies showing expression of the μ3 subtype and coupling to NO release; SH-SY5Y cells, which express the 1.4 kb transcript, do not release NO in response to morphine (data not shown).

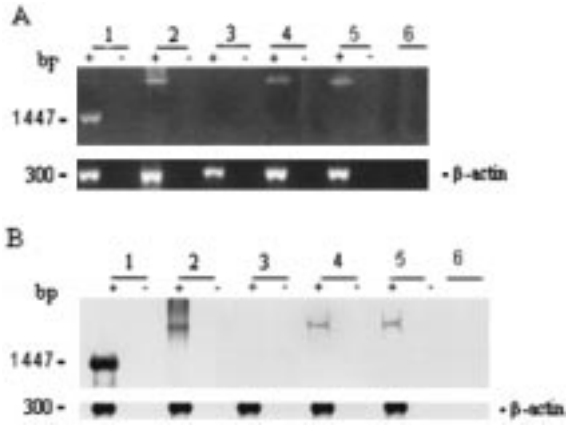

Fig. 2. μ-Specific transcripts expressed in lung and lung carcinoma. (A) Identification of μ opioid receptor transcripts in human lung, lung carcinoma, thyroid and granulocytes by nested RT-PCR. Representative examples of PCR products (agarose gel electrophoresis) are shown. Lane 1, SH-SY5Y cells; lane 2, granulocytes; lane 3, thyroid; lane 4, non-small-cell lung carcinoma; lane 5, normal lung. Samples without reverse transcriptase were included (−) to monitor for genomic contamination while a sample without template (lane 6) was added to monitor PCR contamination. (B) Southern blot hybridization of PCR products. Blotting and hybridization conditions were as described (see Section 2.4). We found comparable results in almost all tissues tested, therefore, one representative sample for each tissue is illustrated. Lane 1, SH-SY5Y cells; lane 2, granulocytes; lane 3, thyroid; lane 4, lung carcinoma; lane 5, normal lung. Samples without reverse transcriptase were included (−) to monitor for genomic contamination while a sample without template (lane 6) was added to monitor PCR contamination.

4. Discussion

NO appears to play complex and diverse roles in cancer [4]. In addition to causing the initial mutagenic event leading to disease, NO has also been implicated, seemingly paradoxically, in both tumor reduction/prevention and in tumor promotion. In this study, we demonstrate that activation of the μ3 opiate receptor by opiate alkaloids in tumor cells leads to a rapid and substantial release of NO. This suggests that endogenous opiate alkaloids may be involved in the upstream signaling events leading to NO production and its subsequent effects on tumor prevention or promotion. Currently, we are investigating the mechanism responsible for the observed increase in opiate-mediated NO release in lung carcinoma. Our results presented here (Fig. 2A,B) suggest that it is not due to increased expression of the μ3 receptor. While nitric oxide synthase (NOS) expression was found not to be upregulated in non-small-cell lung carcinoma [19], recent studies have shown that in vivo, the opioid receptor antagonist naltrexone decreases iNOS mRNA expression in splenocytes [20], suggesting that signaling through opiate receptors can regulate NOS expression.

Previous studies have shown that NO produced in immune cells and endothelial cells is tumoricidal, possibly by inducing apoptosis [21]. Likewise, opioid agonists such as morphine inhibit cell growth of lung cancer cells in vitro and in vivo [5] and the opiate antagonist naltrexone can cause regression of mammary tumors in mice [6]. Our studies suggest that these morphine-induced effects could be mediated by NO through the μ3 receptor. In addition to inhibiting tumor growth, NO may also play a role in

preventing metastatic disease. Metastatic melanoma cells transfected with iNOS show a decrease in their tumorigenic and metastatic potential [22]. Non-tumor host cells may also be able to affect metastasis by inhibiting adhesion of circulating tumor cells [23]. Results from previous studies support a role for morphine as a potential upstream signal in this process. Morphine-mediated NO release induces vasodilation and a reduction in cell adherence [24].

In addition to reducing cancer progression, NO has been shown to have tumor-promoting effects, some of which may be due to an inhibitory effect of NO on the host immune response. NO can decrease T-cell proliferation and may prevent proliferation and infiltration of leukocytes [25]. NO can also decrease adhesion molecule expression, increase vascular permeability and increase angiogenesis [4]. Likewise, morphine can induce morphological changes in immune and endothelial cells through μ3–NO coupling [17,24], resulting in inhibition of chemotaxis, altered phagocytosis and a diminished responsiveness to different cytokines [9]. Our demonstration in this study of μ3 receptor expression in lung and lung carcinoma and our observation of increased NO production in lung carcinoma suggests that tumors could also use endogenous opiates and NO processes to downregulate the host immune response.

In addition to demonstrating functional expression of the μ3 opiate receptor subtype in granulocytes, lung and lung carcinoma, we also show by RT-PCR that these tissues express a specific transcript which is larger in size than the μ receptor expressed in SH-SY5Y cells. Our Southern blot analysis shows that both the 1.4 kb transcript expressed in SH-SY5Y cells as well as the larger ~2.0 kb transcript expressed granulocytes, lung and lung carcinoma hybridize to a probe derived from a single μ gene exon. These results, together with previous results from other laboratories, suggest that the μ receptor subtypes are generated by alternative splicing of a single μ gene [26,27]. Furthermore, our data suggests a correlation between expression of the 2.0 kb transcript in granulocytes, normal lung and lung carcinoma, and production of NO in response to morphine.

In conclusion, this study provides novel experimental findings regarding the expression of the μ3 opioid receptor in humans and its coupling to NO production in lung carcinoma. Further study of the role of endogenous opiates in mediating NO release in tumor cells and continued study of NO processes in cancer progression will lead to a better understanding of the complex and diverse role of this molecule in tumor prevention and tumor promotion.

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