Expression and regulation of PLUNC in human nasal epithelium

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Expression and regulation of PLUNC in human nasal epithelium

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ABSTRACT

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Objectives. PLUNC (palate, lung, and nasal epithelium clone) protein is a member of the bactericidal/permeability-increasing protein (BPI) family, and may have an important role in host defense against bacteria The localization and regulation of PLUNC protein in human nasal epithelium was investigated. First, we located epithelial cells expressing PLUNC protein in the human nasal mucosa. Secondly, we sought to identify PLUNC protein in either human nasal secretions from healthy volunteers or apical secretions from cultured human nasal epithelial cells. Lastly, we investigated whether epithelial differentiation and proinflammatory cytokines influence the expression of PLUNC in human nasal epithelial cells. ***Methods.*** Immunohistochemical staining for PLUNC was conducted on nasal turbinate specimens. Western blot analysis was conducted on nasal secretions from healthy volunteers, apical secretion from cultured human nasal epithelium, and on normal-appearing posterior ethmoid mucosa, inferior turbinate, and nasal polyp specimens. Reverse transcription-polymerase chain reactions of PLUNC were performed with

mRNA from cultured human nasal epithelium cells treated with either interleukin-1β or tumor necrosis factor-α. ***Results.*** PLUNC was expressed in ciliated cells of surface epithelium and serous cells of the submucosal gland in the human nasal mucosa, and was also found in both the nasal secretions of healthy volunteers and apical secretions of cultured human nasal epithelial cells. The degree of mucociliary differentiation and proinflammatory mediators did not influence the expression of PLUNC gene and protein in nasal epithelium. ***Conclusions.*** We demonstrated that PLUNC is secreted from nasal epithelial cells and is not influenced by differentiation or proinflammatory mediators. The functional role of PLUNC in the human airway has yet to be elucidated.

Key words: secreted protein, nasal epithelial cells, PLUNC, host defense

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I. INTRODUCTION

The upper respiratory tract, especially the nasal cavity, is the major route of entry of bacterial pathogens, and when the body recognizes bacterial products, such as lipopolysaccharides (LPS) from gram-negative bacteria, a host defense mechanism takes place. Early and appropriate recognition of bacterial products is critical in maintaining innate immunity and for survival of the host. There are four protein families involved with the host defense mechanism: bactericidal/permeability-increasing protein (BPI), lipopolysaccharide-binding protein (LBP), cholesteryl ester transfer protein (CETP), and phospholipids transfer protein (PLTP)[1]. Among these four protein families, a precise balance of the BPI and LBP family is critical for the host defense mechanism[1,2].

PLUNC (palate, lung, and nasal epithelium clone) protein is a member of the BPI family, and may have an important role in host defense against bacteria[1-3]. The gene responsible for encoding PLUNC protein was recently cloned in the human, although its function remains unknown; PLUNCs expression is specific to nasopharyngeal tissue[4]. Additionally, the amino acid sequence of PLUNC is highly similar to those of secretory proteins produced by salivary glands and the glandular epithelium of the trachea, as well parotid secretory protein. In humans, seven PLUNC proteins have been discovered[1,4]. In the present study, we investigated the localization and regulation of PLUNC in human nasal epithelium by using SPLUNC1, the most widely known protein of the seven. We first examined which type of epithelial cells express PLUNC protein in human nasal mucosa. Secondly, we examined whether PLUNC protein is located in human nasal secretions and apical secretions by analyzing samples from healthy volunteers and from cultured human nasal epithelial cells, respectively. Lastly, we investigated whether the degree of epithelial differentiation or proinflammatory cytokines, such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α), can influence the expression of PLUNC in human nasal mucosa and cultured normal human nasal epithelial (NHNE) cells.

II. MATERIALS AND METHODS

1. Immunohistochemical staining for PLUNC and AB/PAS staining

Human nasal inferior turbinate specimens were harvested and fixed with 4% paraformaldehyde for 24 hours, cryoprotected with 12% and 18% sucrose, and stored in a deep freezer. The specimens were then sectioned (10 μm width), and the frozen sections were stained with a monoclonal PLUNC(SPLUNC1) antibody (R & D systems, Minneapolis, MN). The antigen/antibody reaction was detected with peroxidase-conjugated anti-mouse secondary antibodies. Negative controls were subjected to routine control conditions: omission of primary antibody and use of irrelevant antibody purified mouse IgG.

Adjacent sections were collected on coated slides, treated with 3% glacial acetic acid for three minutes, incubated with Alcian Blue (AB) (pH 2.5) (Muto Chemicals, Tokyo, Japan) for 40 minutes, and lastly with a 0.5% periodic acid solution (Fisher Scientific, Pittsburgh, PA) for 5 minutes followed by a rinse with phosphate-buffered saline. Sections were then reacted with Schiff solution (Sigma Chemical Company, St. Louis, MO) for 10 minutes, washed with tap water, and counterstained with Harris hematoxylin.

2. Air-Liquid Interface (ALI) cultures

Normal human nasal epithelial (NHNE) cells (1 x 10^5cells/culture) were seeded in 0.5 ml of culture medium on a 24.5 mm, 0.45 μm pore size, Transwell-clear (Costar Co., Cambridge, MA) culture insert. Cells were cultured in a 1:1 mixture of basal epithelial growth medium (BEGM) and Dulbecco's modified Eagle's medium (DMEM), containing all the supplements previously described[5]. Cultures were grown submerged for the first 9 days, during which time the culture medium was changed on day 1 and every other day thereafter. The ALI was created on day 9 by removing the apical medium and feeding the cultures from only the basal compartment; the culture medium was changed daily after creation of the ALI. Total RNA was collected as described previously[5] at 2, 7, 14, and 28 days after confluence to determine PLUNC mRNA levels as a function of differentiation.

3. IL-1β and TNF-α treatment of NHNE cells

The passage-2 NHNE cells were grown to confluence in 6 well plates. The cells were treated with either IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 24 hours two days after the generation of the ALI. Total RNA was collected for RT-PCR of *plunc*.

4. Reverse transcription-polymerase chain reaction for PLUNC in IL-1β and TNF-α treated NHNE cells

Oligonucleotide primers were designed according to published sequences for PLUNC (GenBank accession number AF172993, 115 bp, 5' primer: GAT GGC CAC CGT CTC TAT GT; 3' primer: TTT CTG CAG TGA TGT CCA GC). Oligonucleotide amplimers for β2M, which was used as a control gene for RT-PCR, were purchased from Clontech Laboratories (Palo Alto, CA; they generated a 335-bp PCR fragment). RT-PCR was performed using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) according to the manufacturer's recommendations. Total RNA (1 μg RNA per 20 μL reaction volume) was reverse transcribed into complementary DNA (cDNA) using random hexanucleotide primers and the Moloney murine leukemia virus RT. Denaturation, annealing, and extension was carried out at 95°C for 1 minute, 58°C for PLUNC and 55°C for β2M, and at 72°C for 1 minute, respectively. A comparative kinetic analysis was used to compare the levels of mRNA for each gene. PCR products were separated by electrophoresis on a 2% Seakem agarose gel (FMC, Rockland, ME) containing 50 ng/mL ethidium bromide and photographed using Polaroid type 55 film. Negative controls were performed by omitting reverse transcriptase (RT) from the RT reaction in order to verify that the amplified products were indeed from mRNA and not genomic DNA contamination.

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5. Western blot analysis for PLUNC in tissues and nasal secretion

Specimens of normal-appearing mucosa were obtained from the posterior ethmoid sinus with an intranasal ethmoidectomy from 3 patients whose diseases were confined to the anterior ethmoid region. Inferior turbinate mucosa specimens were obtained from 3 patients who had undergone septoplasties. Three nasal polyp specimens were obtained from patients with chronic sinusitis who did not have histories of asthma, aspirin sensitivity, or cystic fibrosis. None of the patients had been on a regimen of intranasal medication, oral steroids, or antibiotic treatment for 3 months prior to the study, and an allergic skin-prick test was negative for all of the patients.

With informed patient consent, nasal secretions from 3 healthy volunteers were collected with sterile syringe and reconstituted with Western lysis buffer [250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% BPB, 10% glycerol]. Secretions from cultured NHNE cells were collected with 1 ml of PBS from each of the 6 Transwell cultures, precipitated with cold 20% trichloroacetic acid (TCA)/acetone solution, and lysed with Western lysis buffer.

Equal amounts of the whole cell lysates from tissue samples and secretion samples were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in Tris-buffered saline [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] at

room temperature for 2 hr, incubated overnight with PLUNC monoclonal antibodies in TTBS (0.5% Tween 20 in TBS), washed with TTBS, and further incubated at room temperature for 1 hr with anti-mouse secondary antibodies (Cell Signaling Tech., Beverly, MA) in TTBS. Blots were visualized using the ECL system (Amersham-Pharmacia, Piscataway, NJ).

III. RESULTS

1. Expression of PLUNC in nasal surface epithelium and submucosal glands

By comparison of the AB/PAS staining and PLUNC expression of the serial sections, we found that PLUNC was mainly expressed in the cytoplasm of AB/PAS-negative ciliated cells and ciliary border of the surface epithelium of nasal turbinate mucosa (Figure 1A, 1B). In the submucosal glands, PLUNC was expressed in AB/PAS-negative serous cells, but not in mucous cells (Figure 1C, 1D).

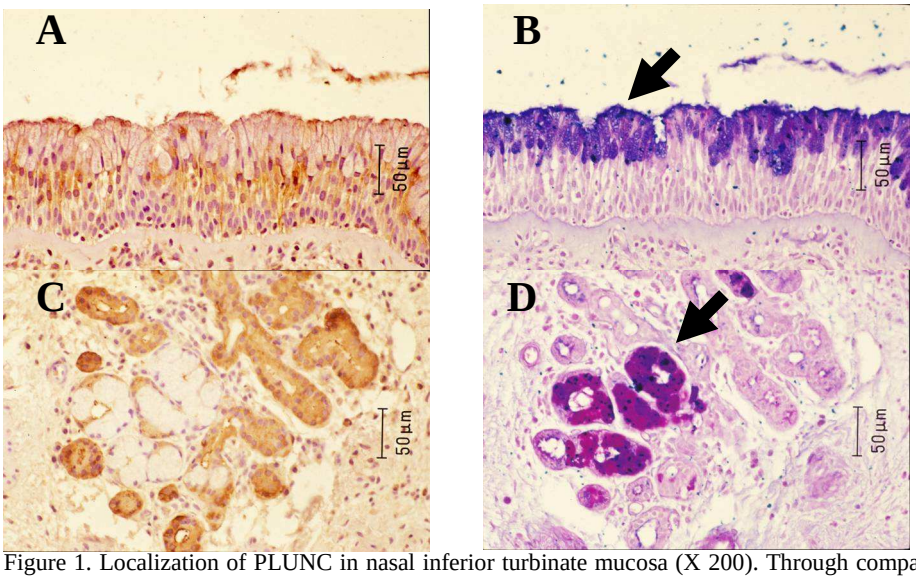

Figure 1. Localization of PLUNC in nasal inferior turbinate mucosa (X 200). Through comparison of the AB/PAS staining and PLUNC expression of serial adjacent sections, PLUNC was mainly expressed in the cytoplasm of AB/PAS-negative non-mucous cells and ciliary border in the epithelial surface (A). Mucous cells were heavily stained with AB/PAS in the surface epithelium (arrow) (B). Among the submucosal gland cells, PLUNC was only expressed in AB/PAS-negative serous gland cells (C). Mucous cells were also heavily stained with AB/PAS in the submucosal gland (arrow) (D).

2. Expression of PLUNC in human nasal secretion and apical secretion from cultured human nasal epithelial cells

PLUNC was detected as a 30 kDa protein from both the nasal secretion of healthy volunteers and apical secretion of cultured NHNE cells (Figure 2). Accordingly, we confirmed that PLUNC is a secreted protein in human nasal mucosa.

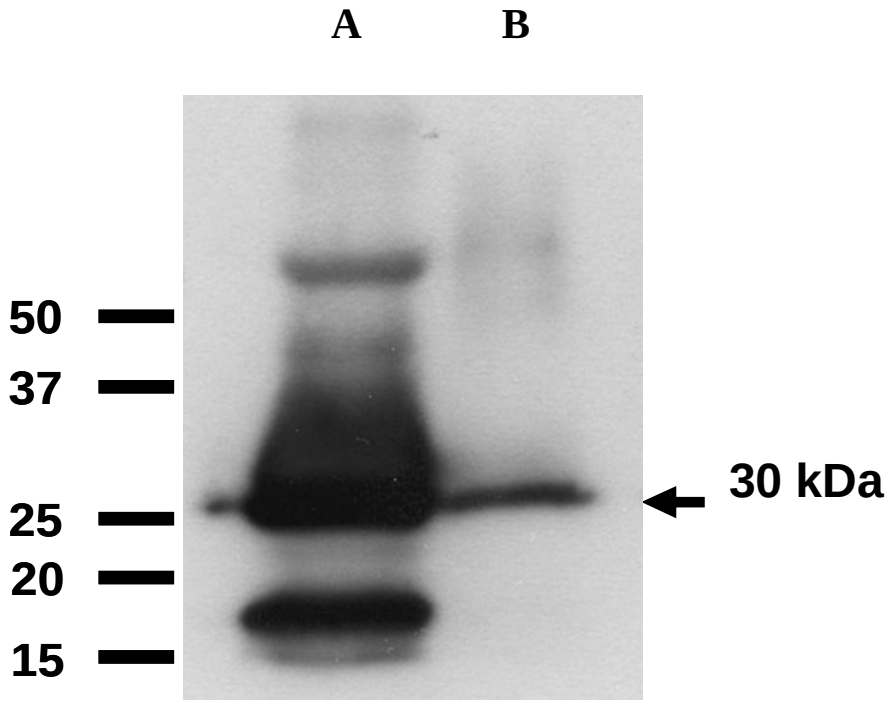

Figure 2. Expression of PLUNC protein in human nasal secretions and apical secretion from cultured human nasal epithelial cells. Secreted PLUNC protein was detected in the nasal secretion of the normal subject *in vivo* (A) and in the apical surface fluid of cultured human nasal epithelial cells *in vitro* by Western blot analysis (B). (PLUNC protein = 30 kDa)

3. PLUNC mRNA expression as a function of differentiation in NHNE cells

In the presence of specific amounts of retinoic acid in the culture medium, mucociliary differentiation is induced[6]. Messenger RNA levels of PLUNC were not altered as a function of mucociliary differentiation in NHNE cells (Figure 3), suggesting that the degree of mucociliary differentiation does not influence the PLUNC expression level in the nasal epithelium. The β2-microglobulin levels show that the amount of mRNA was constant.

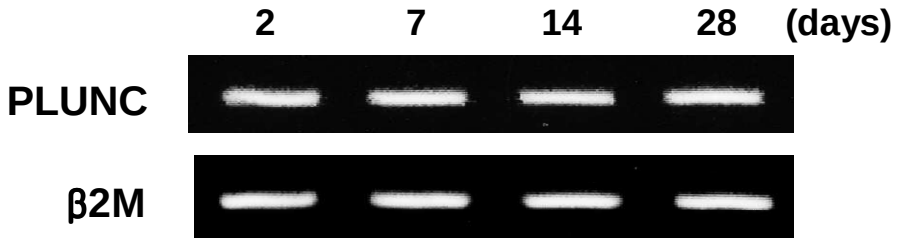

Figure 3. Messenger RNA expression of PLUNC as a function of differentiation. PLUNC expression was constant at 2, 7, 14, and 28 days after confluence

4. Regulation of PLUNC expression by inflammation in cultured NHNE cells and human nasal polyps

Levels of PLUNC mRNA expression were not significantly changed after treatment with IL-1β or TNF-α in NHNE cells (Figure 4A). Moreover, PLUNC expression levels were constant in normal-appearing posterior ethmoid mucosa, which was not directly exposed to any of inflammatory stimulants, inferior turbinate mucosa, which was continuously stimulated by noxious gases, air pollutants, and various inflammatory mediators and nasal polyps, which were exposed to various types of inflammatory stimulants (Figure 4B). Negative controls were performed by omitting reverse transcriptase (RT) from the RT reaction in order to verify that the amplified products were indeed from mRNA and not genomic DNA contamination; no PCR products were observed in the absence of RT.

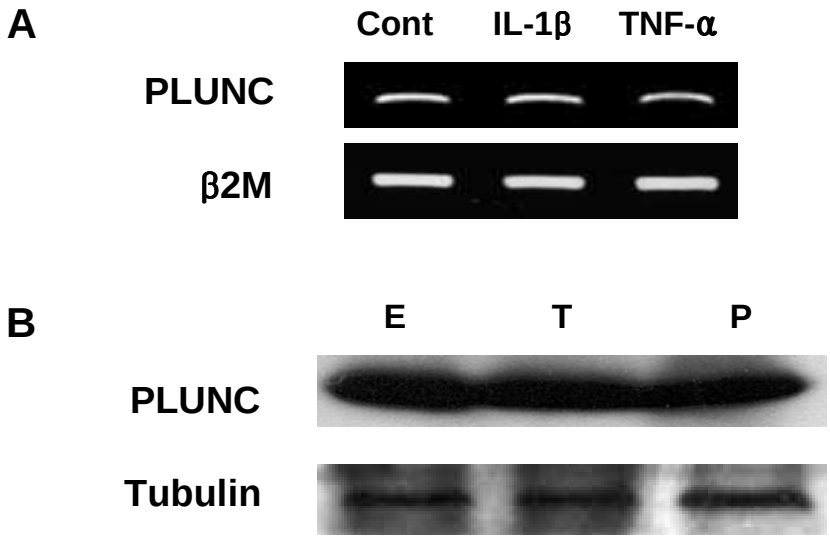

Figure 4. Regulation of PLUNC expression by inflammation. Messenger RNA expression of PLUNC was not affected by inflammatory mediator (IL-1β and TNF-α) treatment in cultured human nasal epithelial cells (A). PLUNC protein expression was not altered by inflammatory status of the nasal tissue (B). (E: normal-appearing posterior ethmoid mucosa, T: inferior turbinate mucosa, P: nasal polyps)

IV. DISCUSSION

Following the identification of *plunc* in the nasal and palatal epithelium of the mouse[7], *plunc* homologues have also been identified in rats[8] and cows[9]. Human studies show that *plunc* is also expressed in the restricted area of nasopharyngeal and tracheobronchial epithelium[4,10]. In humans, the *plunc* gene family encodes 3 short proteins (SPLUNC 1-3) and 4 long proteins (LPLUNC 1-4)[1]. PLUNC, the protein product of human *plunc*, is also known as LUNX[11] and SPURT[12], and has a structure very similar to that of other secreted proteins such as parotid secreted protein and von Ebner minor salivary gland protein[7,10,12]; PLUNC has been identified in nasal secretions[8,13,14], sputum, and saliva[15]. The up-regulation of PLUNC has been reported to occur during inflammatory conditions[12,13] and after olfactory bulbectomy[8].

In the present study, PLUNC was expressed in the cytoplasm of ciliated cells of surface epithelium, which were not stained with AB/PAS (Figure 1A, 1B), and we speculate that ciliated cells actively secrete PLUNC in the surface epithelium; we were also able to identify secreted PLUNC in the ciliary border of the surface epithelium (Figure 1A). In submucosal glands, PLUNC was also expressed in serous cells, but not in mucous gland cells (Figure 1C, 1D). These results are consistent with other report that human PLUNC was expressed in the serous cells in the tracheobroncheal and lung tissue[16].

PLUNC protein was detected with Western blot analysis as a 30 kDa protein in both human nasal secretions from healthy volunteers and apical secretions of

cultured NHNE cells. These data support previous findings showing that PLUNC protein is a secreted protein present in nasal secretion[8,13,14] and in apical secretion of cultured human tracheobronchial epithelia cells[12]. However, we showed for the first time that PLUNC was also secreted in apical secretion of cultured NHNE cells.

To date, the regulation of PLUNC protein has not yet been fully elucidated. In this study, we treated NHNE cells with IL-1β and TNF-α, which are the most important proinflammatory mediators present in the airway. Levels of PLUNC gene expression (Figure 3A) and protein expression (data not shown) were not significantly altered following treatment in NHNE cells. Moreover, PLUNC protein expression was not affected by various inflammatory status of the nasal tissue *in vivo* (Figure 3B). These findings suggest that inflammatory mediators may not regulate the PLUNC expression. Additionally, the degree of mucociliary differentiation did not influence PLUNC expression in cultured NHNE cells. These results suggest that PLUNC protein was secreted constantly even in early and late stage of mucociliary differentiation. Consequently, because the expression of PLUNC protein is not dynamic, we do not consider it problematic to conduct *in vitro* studies on PLUNC expression on the early period of culture after confluence.

Our findings were inconsistent with previous reports that PLUNC expression is up-regulated by DMBA (dimethylbenzylamine, chemical irritant) inhalation in epoxy-worker's nasal lavage fluid but not in healthy subjects[13]. In addition, Wu et al. report that PLUNC gene expression is up-regulated more in tracheobroncheal tissue of patients with chronic obstructive pulmonary disease than in healthy subjects using an

in situ hybridization method[12]. The reason for this discrepancy is not clear at this time; however, it suggests that PLUNC expression may be selectively regulated within different tissue environments.

V. CONCLUSION

In conclusion, we show that PLUNC protein is secreted from nasal epithelial cells and is not influenced by the mucociliary differentiation and proinflammatory mediators. The functional role of PLUNC in the human airway has yet to be elucidated.

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ABSTRACT (IN KOREAN)

사람 코점막 상피에서 PLUNC의 발현 및 조절

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김규보

사람 코점막 상피에서 PLUNC 의 존재 및 분비조절에 대해 알아보고자 하였다. 첫째, 정상 사람 코점막의 어떤 상피 세포에서 PLUNC 단백이 발현되는지 알아보았고, 둘째, 건강한 지원자의 비강 분비물 및 배양된 사람 코점막 상피세포(cultured human nasal epithelial cells)의 위쪽 분비물에 PLUNC 단백이 존재하는지 확인하였으며, 셋째, 염증 싸이토카인이 사람 코점막 상피세포 및 배양된 사람 코점막 상피세포에서 PLUNC 의 발현에 영향을 미치는지 알아보았다. 연구방법으로는 사람 하비갑개 점막에 PLUNC 항체로 면역조직화학염색을 시행하였으며, 건강한 지원자의 비강 분비물, 배양된 코점막 상피세포의 위쪽 분비물 및 정상적으로 보이는 후사골동 점막, 경도의 염증 소견이 있는 하비갑개 점막 및 비용 검체를 대상으로 Western blot analysis 을 시행하였다. 또한, 역전사중합연쇄반응(RT-PCR)을 이용하여 interleukin-1ß(IL-1ß) 또는 tumor necrosis factor-α(TNF-α)로 처치한 배양된 사람 코점막 상피세포에서 PLUNC mRNA 가 발현이 되는지 확인하였다. PLUNC 는 표면 상피세포 중 섬모 세포와 점막하 샘의 장액 세포에서 발현되었으며, 건강한

지원자의 비강 분비물 및 배양된 사람 코점막 상피 세포의 위쪽 분비물 모두에서 발견되었다. 그러나 점액섬모 분화도나 염증매개물질은 PLUNC 유전자와 단백의 발현에 영향을 끼치지 않았다. 따라서, PLUNC 단백이 코점막 상피 세포에서 분비는 되나 분화도나 염증매개물질의 영향을 받지 아니함을 보여주었으며, 앞으로 PLUNC 의 기능적인 역할이 밝혀져야 할 것으로 사료된다.

핵심되는 말 : 분비단백, 코점막상피세포, PLUNC, 인체 방어 작용