I. INTRODUCTION

Oxygen is a potent and essential signaling molecule in cells, and hypoxia, defined as a state where oxygen tension drops below normal limits, plays a pivotal role in pathological conditions, including tumors. Hypoxia can restrict tumor growth and tumors with poor vascularization fail to grow, leading to apoptosis. In contrast, certain types of cancer cells can survive and even proliferate under hypoxic conditions by triggering specific cellular and systemic adaptive responses.

These processes contribute to the selection for more aggressive and metastatic tumor phenotypes that are associated with poor prognosis. Both normoxic and hypoxic cells require stimuli from extracellular environment, and the stimuli are transduced through various signaling pathways. Among numerous signaling pathways, PI3K/Akt and Raf/MEK/Erk pathways are...
known as major routes in survival and progression of tumor cells, and activation of these pathways may be important for survival of cancer cells exposed to hypoxia. Little has been known regarding the Raf/Erk signaling pathway involved in activation of these kinases and their potential roles in hypoxic cancer cells, whereas PI3K/Akt pathway has been studied by numerous investigators. Raf/MEK/Erk pathway is an evolutionary conserved pathway that conveys signals from cell surface receptors to ERK/MAPK. Because Raf-1 is a vital component of a variety of growth factor–induced signaling pathways, stimulation of the MEK–ERK pathway and inhibition of death signaling through its kinase-dependent and independent mechanisms may ensure cell survival and proliferation. Therefore, response of Raf /MEK /Erk pathway may be one of the major determinants in cell survival/apoptosis under hypoxic condition.

Seko et al observed that hypoxia caused rapid activation of Raf–1 in cultured rat cardiac myocytes. In addition, ERK activation has been reported to be enhanced after ischemia in vivo. However, there are few reports on relation between Raf/Erk pathway and hypoxia, and it is not clear whether Raf /Erk pathway is stimulated by hypoxia, in cancer cells. The present study was, therefore, undertaken to investigate whether the Raf /ERK signaling pathway is activated by hypoxia and, if so, to determine their activator in oral squamous cell carcinoma (OSCC) cells.

II. MATERIALS AND METHODS

1. Cell culture

A YD8 cell line was established from the cancer of an oral squamous cell carcinoma (OSCC) patient and were gifted from the Department of Oral Pathology, College of Dentistry, Yonsei University (Seoul, Korea). The cells were grown in RPMI 1640 medium (Gibco, Rockville, MD) with 10% fetal bovine serum (Gibco) and incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

2. Reagents

PD168393 (EGFR inhibitor) was obtained from Calbiochem (La Jolla, CA), and cobalt chloride was purchased from Sigma (St. Louis, MO). The antibodies against phospho–PTEN (Phosphatase and TEnsIn ho–molog), phospho – PDK1 (phosphoinositide dependent kinase), PTEN, PDK1, and ILK (integrin–linked kinase) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho–Erk, phosphor – Raf – 1, Erk, Raf – 1, and B – raf were purchased from Santa Cruz (Santa cruz, CA). The goat anti–actin antibody, anti–rabbit IgG antibody and rabbit anti–mouse IgG antibody were also obtained from Santa Cruz.

3. Western blot analysis

YD8 cells were lysed in lysis buffer (150 mM NaCl, 0.2% Nonidet P–40, 50 mM NaF, 100 μM Na₃VO₄, pH 7.2) in the presence of protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) on ice for 30 min, followed by clarification by centrifugation at 12,000 g for 15 min. Protein concentrations were determined using the Bio–Rad Protein assay (Bio–Rad, Hercules, CA). Proteins (50 g) were resolved on 10% poly–acrylamide gels and transferred onto polyvinylidene fluoride membranes (Bio–Rad). Membranes were blocked with 5% non–fat milk in Tris–buffered saline (TBS, pH 7.6) containing 0.1% Tween (TBST) for 1 h at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies at 4°C. The membranes were washed in TBST and were incubated with the respective horseradish peroxidase–conjugated secondary antibody in TBST. After washing in TBST, bound secondary antibodies
were detected using Supersignal West Femto substrate (Pierce, Madison, WI).

III. RESULTS

1. Identification of an Akt activator in OSCC cells under hypoxic condition induced by CoCl₂

Previously we studied Akt activation by hypoxia using CoCl₂, a hypoxia-mimicking agent, and observed increased Akt phosphorylation. To identify which upstream molecule is involved in Akt activation, YD 8 cells were treated with 0, 10, 50, 100, 200, or 300 μM of CoCl₂ for 3 h. Then the activities of PTEN and PDK1 were assessed via immunoblotting using specific antibodies raised against phosphorylated forms of those proteins. In addition, the expression level of ILK, which is known as a possible Akt activator, was checked. Treatment with CoCl₂ resulted in no apparent change in phosphorylation pattern of PTEN and PDK1 as well as total expressions. The level of ILK protein was not affected by CoCl₂, too (Fig. 1).

2. CoCl₂ activates Erk in a Raf kinase-independent mechanism

We examined whether CoCl₂, a hypoxia-mimetic agent, induced activation of Erk, a kinase promoting cell survival, YD 8 cells were treated with various concentrations of CoCl₂ for 3 h, and the activation of ERK was estimated by the levels of phosphorylation of this kinase. Treatment with CoCl₂ resulted in an appreciable up-regulation of protein expression of phosphorylated Erk in YD 8 cells in a dose-dependent manner, except in cells treated with 200 μM of CoCl₂ (Fig. 2A). Next, to determine the time course of CoCl₂ effect on Erk activation, cells were treated with 300 μM of CoCl₂ for time periods from 0 h, 3 h, and 6 h. As shown in Fig. 2 B, the level of active phosphorylated Erk began to increase 3 h after CoCl₂ treatment. We also attempted to investigate the molecular mechanism by which CoCl₂ induces Erk activation in OSCC cells. The level of Erk phosphorylation is mainly dependent on Raf kinase activity, since Erk phosphorylation is invariably stimulated by active Raf kinase. Though Erk phosphorylation was significantly increased by CoCl₂, there was no change in both Raf–1 kinase activity and its protein expression. The level of B–raf protein was also not affected by CoCl₂ treatment. In addition, longer treatment of CoCl₂ did not evoke Raf activation (Fig. 3).

3. Erk activation by CoCl₂ treatment occurs through EGFR

In a previous report increased Akt phosphorylation by CoCl₂ indicates that the hypoxic condition provides survival signals to cells through epidermal growth factor receptor (EGFR). To clarify whether EGFR is also involved in CoCl₂-induced Erk activation, we pretreated YD 8 cells with 1 μM of PD168393, an EGFR inhibitor, for 1 h and then added 300 μM of CoCl₂ to the cells for 3 h. CoCl₂-stimulated Erk phosphorylation was greatly inhibited by PD168393, suggesting that the phosphorylation of Erk is EGFR-dependent. Raf kinase was not inhibited by PD168393 as expected (Fig. 4).

4. Activities of PTEN and PDK1 is independent on EGFR signaling

To investigate the possibility of involvement of EGFR signaling in activities of PTEN and PDK1, YD 8 cells were treated with the same as above-mentioned procedure, and the expression level of active dephosphorylated PTEN and phosphorylated PDK1 proteins was observed. Pretreatment of 1 μM of PD168393 to YD cells had no role in phosphorylations of PTEN and PDK1. The level of ILK protein was not changed by the EGFR inhibitor, too (Fig. 5).
IV. DISCUSSION

For Akt to be activated, Akt requires phosphatidylinositol-3,4,5-triphosphate (PIP3) of cell membrane, and the PIP3 is made by phosphoinositide 3-kinase (PI3K). The activity of PI3K is regulated by numerous factors, such as PDK1, PTEN, and cytokines. Phosphorylated PDK1 is an active form of PDK1, which activates Akt kinase by phosphorylating PI3K, whereas PTEN stimulates PI3K/Akt pathway when it is dephosphorylated. It is known that both PDK1 and PTEN are major determinants in PI3K/Akt activity.

However, in this study it does not seem that PDK1 and/or PTEN are involved in CoCl2 induced Akt activation. In addition, ILK is not a direct activator of Akt in our results, though ILK is strongly suggested as a possible candidate for PDK2 activating PI3K/Akt pathway. Further study is needed to define a specific upstream molecule in CoCl2 induced Akt activation.

Although hypoxia is a injurious stimulus to cells and usually result in cell death, it also mediates a number of adaptive biological response and regulates the expression of a variety of genes that are involved in cell survival.
ine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation, and apoptosis. The extracellular signal–regulated kinase (ERK) is a subfamily member of MAPks. Previous studies have shown that hypoxia stimulates Erk activation contributing to cell survival.\(^6\)-\(^8\) Consistent with these reports, we observed that treatment of OSCC cells with CoCl\(_2\), a well-known hypoxia-mimetic agent, activated Erk. In contrast to our data and the data of others, a few studies have shown that hypoxia suppressed rather than increased the Erk activation\(^12\). The authors propose that Erk activation induced by hypoxia-mimicking CoCl\(_2\) might depend on cell type and not a general effect of hypoxia.

Erk is activated by an upstream kinase called MAPK/ERK kinase (MEK) in response to growth stimuli. The MEK is stimulated by Raf gene family, consisting of of A–Raf, B–Raf and Raf–1. Accordingly, Erk activity is mainly regulated by the Raf kinase. Our study showed no change in Raf–1 kinase activity implying that CoCl\(_2\) induces Erk activation in Raf–1 kinase independent mechanism. Several studies indicate that B–raf might be the predominant activator of MEK and that Raf–1 has another role in protection against apoptosis, which does not require its kinase activity or its ability to activate MEK\(^13\). Taken together, it is suspected that Erk activation may occur through B–raf kinase when CoCl\(_2\) induces Erk phosphorylation. The authors are planning to confirm the role of B–raf kinase in CoCl\(_2\) –induced Erk activation.

The Erk signaling pathway has been reported to be activated by growth factor receptors including EGFR.\(^4\),\(^15\). To investigate if Erk activation by chemically induced hypoxic condition in this study is dependent on EGFR, we examined the effect of the specific EGFR inhibitor PD168393. The phosphorylation of the Erk kinase was inhibited by the inhibitor, and this finding suggests that hypoxia induces Erk activation through a EGFR–dependent mechanism. The Erk activation occurs not through EGFR/Raf pathway, though numerous studies support the idea that activation of Erk is primarily mediated by Raf kinase. Furthermore, EGFR signaling also does not contribute to the PDK1 and/or PTEN related PI3K/Akt pathway, based on the finding of no effect of PD168393 on phosphorylation pattern of PDK1 and PTEN.

In conclusion, the present study shows that CoCl\(_2\)-induced hypoxia causes activation of Erk survival signal in OSCC cells through EGFR dependent mechanism. However, the Erk activation is not mediated the Raf cascade. The authors of this study propose by that OSCC cells are resistant to hypoxia and attempt to remain alive under this condition by activating Erk through EGFR.

V. REFERENCES


