of elderly patients, although the study relied on self-reporting and review of medical records.[11] Only a few studies have been reported in Japan; the incidence was 12.7% in elderly inpatients of the geriatric unit of University of Tokyo Hospital.[9] In the present survey, the average incidence was 9.2%, ranging from 6.6 to 15.8% among facilities, but was similar to that reported previously.[9] Although the incidence varied among hospitals, it is important to note that the incidence of ADR was more than 5% in all hospitals.

Adverse drug reactions were judged by attending physicians in this study, whereas they were determined by objective review of the medical records in addition to judgment by attending physicians in the previous report from the geriatric unit of University of Tokyo Hospital. In the present study, the incidence of ADR in this facility was 8.8%, which was 30% lower than that in our last survey. This difference may be attributable to underestimation by the attending physicians rather than a decrease in ADR over this short period of 3 years. Therefore, if another authorized person judged the ADR strictly, the overall incidence rate might have been slightly higher.

Our results on the incidence of ADR in elderly patients may add important information. However, all the facilities in this survey were geriatric units of university hospitals, where most of the inpatients were older than 65 years and the doctors in those units are careful in prescribing medication to elderly patients. Therefore, our data might not be directly applicable to elderly patients in other hospitals or units. In fact, ADR were found in nearly half of elderly inpatients of the neuropsychiatry unit of University of Tsukuba Hospital (unpubl. obs, Mizukami *et al.*). In addition, our data in university hospitals, which are acute care hospitals, might not be applicable to chronic care facilities such as long-term care facilities. Since the introduction of the fixed payment system, Diagnosis Procedure Combination system, to university hospitals in Japan in 2003, drug treatment in university hospitals might be changing in the future. Therefore, the incidence of ADR in various types of hospitals in Japan needs to be studied.

In this study, depression and apathy were found to be associated with ADR in addition to the accumulation of diseases and geriatric syndromes, polypharmacy, an increase of prescribed drugs during hospitalization, longer hospital stay and emergency admission. This result is consistent with other reports.[9] However, the causal relationship remains unknown. A higher number of diseases or geriatric syndromes can lead to an increase in ADR through polypharmacy[8,9] while ADR themselves may increase diseases or geriatric syndromes. Similarly, longer hospital stays can increase the risk of ADR, while ADR prolong the duration of hospitalization. The latter point is critical to medical economics as well. Age was not associated with ADR in this study, inconsistent with other studies. This might be due to effects of education on pharmacotherapy in elderly patients for several years at university hospitals. Although we did not analyze the types or classes of ADR in this survey, it has been reported that severe ADR such as neuropsychiatric disorders or cardiovascular injury occur in elderly patients.[9]

Recently, evidence has been accumulating on drug therapy in the elderly. However, there are very few data available in people aged 75 years and older or in frail elderly people. Therefore, it is necessary to establish the safety and effectiveness of drug therapy in these patients in the future. Evidence-based medicine in the elderly aims to discontinue unnecessary drugs and to avoid polypharmacy. On the other hand, a fixed payment system such as the long-term care insurance system in Japan forces doctors to reduce prescribed drugs from a business viewpoint. Indeed, it has been reported that 0.6 drugs were on average discontinued within a month after admission to long-term care facilities, although adverse drug withdrawal events were very few.[12] Because minimally prescribed drugs have not increased ADR in patients with dementia and a low capacity for medication management,[13] it is necessary to cut down unnecessary drugs in frail elderly patients based on evidence-based medicine. In the USA, Beers' criteria are available to identify potentially inappropriate medication use, in order to reduce drug-related problems.[14] In Japan, however, we do not have such guidelines for drug treatment in the elderly. Because the drugs and medical situation in Japan are different from those in the USA, we need to establish our own guidelines, which will be published this year. In addition, we need to accumulate clinical evidence to support the guidelines. We also need to utilize pharmacists more efficiently, because they are an underused resource in avoiding medication errors and can provide important safeguards for elderly patients in hospitals and nursing homes.

Elderly patients are exposed to more medications and have an increased risk of ADR, many of which are avoidable. Knowledge of pharmacological principles and age-related effects on pharmacokinetics/pharmacodynamics is essential to promote safe prescribing. Other factors related to ADR such as polypharmacy, long admission and depression should also be evaluated during hospitalization.

Acknowledgments

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Renin-Angiotensin System Modulates Oxidative Stress–Induced Endothelial Cell Apoptosis in Rats

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Abstract—The role of the renin-angiotensin system in oxidative stress–induced apoptosis of endothelial cells (ECs) was investigated using a rat model and cultured ECs. EC apoptosis was induced by 5-minute intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 and was evaluated at 24 hours by chromatin staining of *en face* specimens with Hoechst 33342. Although activity of angiotensin-converting enzyme in arterial homogenates was not increased, administration of an angiotensin-converting enzyme inhibitor temocapril for 3 days before H_2O_2 treatment inhibited EC apoptosis, followed by reduced neointimal formation 2 weeks later. Also, an angiotensin II type 1 (AT1) receptor blocker (olmesartan) inhibited EC apoptosis, whereas angiotensin II administration accelerated apoptosis independently of blood pressure. Next, cultured ECs derived from a bovine carotid artery were treated with H_2O_2 to induce apoptosis, as evaluated by DNA fragmentation. Combination of angiotensin II and H_2O_2 dose-dependently increased EC apoptosis and 8-isoprostane formation, a marker of oxidative stress. Conversely, temocapril and olmesartan reduced apoptosis and 8-isoprostane formation induced by H_2O_2, suggesting that endogenous angiotensin II interacts with H_2O_2 to elevate oxidative stress levels and EC apoptosis. Neither an AT2 receptor blocker, PD123319, affected H_2O_2-induced apoptosis, nor a NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, influenced the effect of temocapril on apoptosis in cell culture experiments. These results suggest that AT1 receptor signaling augments EC apoptosis in the process of oxidative stress–induced vascular injury. (***Hypertension.* 2005;45:1188-1193.**)

Key Words: angiotensin ■ apoptosis ■ carotid arteries ■ endothelium ■ free radicals

Stress-induced injury of vascular endothelial cells (ECs) is considered to be an initial event in the development of atherosclerosis.[1] In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking, as well as hypertension, diabetes, and ischemia reperfusion.[1–3] This notion is supported by the findings that the production of reactive oxygen species is upregulated in vascular lesions[4,5] and that lesion formation such as endothelial dysfunction is accelerated by superoxide anion[6] and, in contrast, is attenuated by free radical scavengers, including vitamin E[7] and superoxide dismutase.[8]

The renin-angiotensin system (RAS) is known to play a pivotal role in the process of vascular lesion formation such as atherosclerosis and restenosis after angioplasty. The expression of RAS components renin,[9] angiotensinogen,[10] angiotensin-converting enzyme (ACE),[11,12] and angiotensin II (Ang II) receptors[13] is upregulated in vascular lesions. Also, RAS inhibitors attenuate neointimal formation after vascular injury in animals[12,14] and endothelial dysfunction in humans.[15,16] The interaction between oxidative stress and the RAS, factors essential for the development of vascular disease, needs to be addressed. It has been demonstrated that RAS activation induces oxidative stress[17–20] and can enhance EC apoptosis in vitro.[20,21] However, it has not been elucidated whether the RAS plays a role in oxidative stress–induced vascular injury in vivo, particularly in EC apoptosis, an initial and important process in atherosclerosis.[1,22,23]

In this study, we first tested whether the RAS would augment EC apoptosis induced by brief exposure to H_2O_2 and the subsequent neointimal formation using a rat model.[24] Next, we used an in vitro model of H_2O_2-induced EC apoptosis to clarify the underlying cellular mechanism.

Methods

H_2O_2 Treatment of Carotid Artery

Ten- to 12-week-old male Wistar rats (Japan Clea; Tokyo, Japan) were used in this study. Maintenance of rats and surgical procedures for H_2O_2 treatment were performed as described previously.[24] Methods are detailed in the online data supplement (available online at http://www.hypertensionaha.org). All of the experimental protocols were approved by the animal research committee of the Kyorin University School of Medicine.

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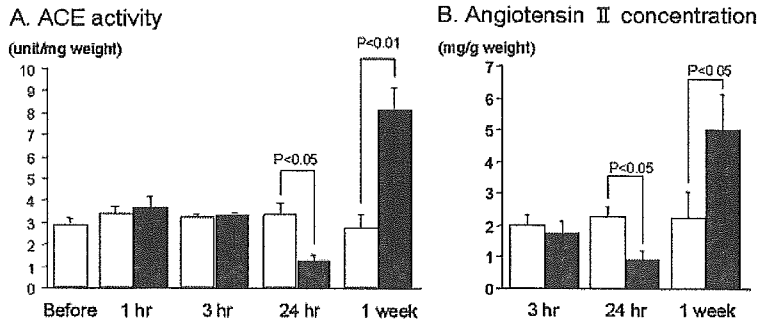

Figure 1. ACE activity and Ang II concentration in rat carotid artery after H_2O_2 treatment. Treated (closed bars) and contralateral (open bar) carotid arteries were harvested at the indicated time points after H_2O_2 treatment. ACE activity and Ang II concentration in tissue homogenates were measured using a pool of samples consisting of 6 to 10 arteries and were calibrated by the tissue wet weight. Values are expressed as mean±SEM of 5 to 6 independent pools.

Animal Groups and Blood Pressure Measurement

An ACE inhibitor, temocapril (10 mg/kg per day; donated by Sankyo Co, Ltd; Tokyo, Japan), or vehicle (40% ethanol) was administered orally using a feeding tube daily for 3 days. Separately, an Ang II type 1 (AT1) receptor blocker, olmesartan (1 mg/kg per day; donated by Sankyo Co, Ltd), or vehicle (40% ethanol) was administered orally for 3 days. Ang II was administered for 3 days using an osmotic minipump (Model 103D; Alza Corporation) prefilled with Ang II (0.7 mg/kg per day; Sigma), and implanted subcutaneously in the back. Hydralazine (25 mg/kg per day; Sigma) was orally administered alone for 5 days and subsequently with or without Ang II for 3 days before H_2O_2 treatment to abolish the effect of Ang II on blood pressure. On the last day of drug administration, blood pressure was measured with the animals in a conscious state by the tail-cuff method (BP-98A; Softron), and then H_2O_2 treatment was performed.

Measurement of ACE Activity and Ang II Concentration

At various time points after H_2O_2 treatment, the carotid arteries were dissected, weighed, and stored at −80°C. Pooled samples (n=6 to 10 for a pool) were homogenized with a polytron homogenizer in distilled water and centrifuged at 25 000*g* for 30 minutes at 4°C. ACE activity and Ang II concentration in the supernatants were measured using a colorimetric assay[12] and a sensitive radioimmunoassay, respectively. The values were calibrated by the tissue wet weight. ACE activity in the cell lysates of cultured ECs was measured using a colorimetric assay and calibrated by the protein concentration.

Evaluation of EC Apoptosis and Neointimal Formation in Carotid Artery

EC apoptosis was evaluated at 24 hours after H_2O_2 treatment as described previously.[24] Neointimal formation in the common carotid artery was evaluated 2 weeks after H_2O_2 treatment as described previously.[24] Methods are detailed in the online data supplement.

Induction of EC Apoptosis in Culture

ECs isolated from bovine carotid artery[25] were used at the fifth to seventh passage. When the cells had grown to 80% confluence, ECs were pretreated for 24 hours with culture medium containing the reagents that were tested in the experiments. Subsequently, after washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.01 to 0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 hours at 37°C to induce apoptosis. The cells were washed twice with Hank's balanced salt solution and then cultured in culture medium containing the reagents until assay.

The effects of temocapril, olmesartan, a NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), an Ang II type 2 (AT2) receptor blocker, PD123319 (Research Biochemical International), and Ang II (Sigma) were examined by adding them into the medium throughout the experiments.

Measurement of EC Apoptosis and Oxidative Stress Markers in Culture

For quantitative determination of apoptosis, we measured DNA fragmentation and caspase-3 activity at 24 hours after H_2O_2 treatment. DNA fragmentation was evaluated by histone-associated DNA fragments using a photometric enzyme immunoassay (EIA; Cell Death Detection ELISA; Roche) according to manufacturer instructions. Caspase-3 activity was measured using a colorimetric kit (Caspase-3 Colorimetric Activity Assay Kit; Chemicon) based on its activity to digest the substrate DVED according to manufacturer instructions.

Formation of 8-isoprostane (8-*iso* prostaglandin $F_{2\alpha}$) was measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were diluted with EIA buffer when necessary and were applied to EIA according to manufacturer instructions. Intracellular oxidative stress levels were measured using 2′,7′-dichlorofluorescein (DCF) as described previously,[26] and the intensity values were calculated using the Metamorph software.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) to quantify AT1 receptor mRNA in cultured ECs was performed using SYBR Green I (Sigma) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Methods are detailed in the online data supplement.

Data Analysis

The values are expressed as mean±SEM in the text and figure data were analyzed using 1-factor ANOVA. If a statistically significant effect was found, Newman–Keuls test was performed to isolate the difference between the groups. Differences with a value of $P<0.05$ were considered statistically significant.

Results

ACE Activity in Carotid Artery After H_2O_2 Treatment

We examined whether H_2O_2 treatment would activate ACE and stimulate Ang II synthesis in the carotid artery. As shown in Figure 1A, ACE activity in tissue homogenates was not increased at 1 to 3 hours and, rather, was decreased at 24 hours, probably because of EC denudation.[24] Low ACE activity in the de-endothelialized artery is consistent with the previous finding[11,12] and was confirmed by measurement of ACE activity in the rat carotid artery, in which ECs were denuded ex vivo using a cotton swab (data not shown). In contrast, ACE activity was significantly increased at 1 week after H_2O_2 treatment, reflecting neointimal formation.[11,12,24] Ang II concentration in arterial homogenates showed similar changes to ACE activity after H_2O_2 treatment (Figure 1B).

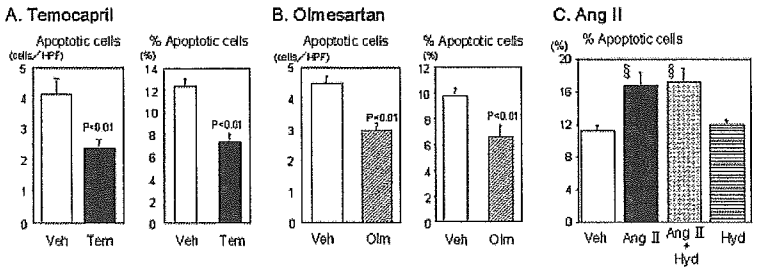

Figure 2. Effects of temocapril (A), olmesartan (B), and Ang II (C) on EC apoptosis after H_2O_2 treatment in rat carotid artery. The number of apoptotic ECs was counted per high power field (HPF; ×200), and the ratio of the apoptotic cell number to the intact cell number was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. A and B, Temocapril (Tem; 10 mg/kg per day; n=12), olmesartan (Olm; 1 mg/kg per day; n=8), or their vehicle (Veh; n=10 and n=6, respectively) was administered orally for 3 days before H_2O_2 treatment. C, Ang II (0.7 mg/kg per day) or its vehicle was administered subcutaneously for 3 days using an osmotic minipump alone (n=8 for Ang II and n=10 for vehicle) or in combination with oral administration of hydralazine (Hyd; 25 mg/kg per day; n=6 for Ang II and n=6 for vehicle; single administration for 5 days and coadministration with Ang II for 3 days) before H_2O_2 treatment. §$P<0.01$ vs vehicle. Values are expressed as mean±SEM.

Effect of RAS Inhibitors and Ang II on EC Apoptosis After H_2O_2 Treatment in Rats

The effects of an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, on EC apoptosis were examined at 24 hours after H_2O_2 treatment because the peak of apoptosis was observed at 6 to 24 hours.[24] Administration of 10 mg/kg per day temocapril or 1 mg/kg per day olmesartan for 3 days before H_2O_2 treatment did not significantly change body weight, heart rate, or blood pressure, but this dose of temocapril effectively inhibited plasma ACE activity (data not shown). The number and percentage of apoptotic cells, as determined using *en face* specimens with Hoechst 33342 staining, were significantly decreased by temocapril compared with vehicle (Figure 2A; supplemental Figure I, available online at http://www.hypertensionaha.org). Olmesartan showed a comparable inhibitory effect on EC apoptosis (Figure 2B).

Ang II was administered for 3 days in combination with hydralazine to eliminate the effect of Ang II on blood pressure. Consequently, systolic blood pressure was higher in rats administered Ang II alone (161±5 mm Hg; $P<0.01$) than in the other groups of rats: 123±3 mm Hg in the vehicle group, 129±7 mm Hg in the Ang II plus hydralazine group, and 114±4 mm Hg in the hydralazine group. In contrast to RAS inhibitors, Ang II administration augmented EC apoptosis independent of the pressor effect because coadministration of hydralazine did not influence EC apoptosis (Figure 2C).

Inhibitory Effect of Temocapril on Neointimal Formation

We examined whether inhibition of EC apoptosis by temocapril would result in a reduction of neointimal formation. To do so, histological analysis of the carotid artery was performed 2 weeks after H_2O_2 treatment. Temocapril significantly decreased the neointimal area and the intima/media area ratio: intima/media area ratio was 0.18±0.02 in the vehicle group versus 0.12±0.02 in the temocapril group (n=9; $P<0.05$; supplemental Figure II). Because temocapril was administered for only 3 days before H_2O_2 treatment, it is suggested that inhibition of EC apoptosis may play a mechanistic role in attenuation of neointimal formation, although ACE inhibitors have various effects such as anti-inflammation and antimigration as well.

Effect of RAS Inhibitors on H_2O_2-Induced EC Apoptosis in Culture

To reproduce oxidative stress–induced EC apoptosis in culture, we applied 0.2 mmol/L H_2O_2 to cultured ECs derived from a bovine carotid artery for 1.5 hours based on dose- and time-response experiments. EC apoptosis, as determined by DNA fragmentation and caspase-3 activity, was induced at 24 hours after H_2O_2 treatment. Comparable to in vivo experiments, temocapril inhibited EC apoptosis in a dose-dependent manner (Figure 3A and 3B). The inhibitory effect on EC apoptosis was mimicked by 10 μmol/L olmesartan (Figure 3C), but an AT2 receptor blocker, PD123319, did not influence EC apoptosis (supplemental Figure IIIA). The involvement of NO in the effect of temocapril was examined using an NO synthase inhibitor, L-NAME, because ACE inhibitors stimulate NO production via the inhibition of bradykinin degradation.[12] However, L-NAME did not influence the effect of temocapril (supplemental Figure IIIB).

To make the interaction between H_2O_2 and Ang II clear, dose response and combined effects of both agents on EC apoptosis and 8-isoprostane formation, a marker of oxidative stress, were examined. As shown in Figures 3D and 4A, combination of Ang II and H_2O_2 dose-dependently stimulated EC apoptosis and 8-isoprostane formation. Conversely, temocapril and olmesartan restrained 8-isoprostane formation (Figure 4B) and intracellular DCF formation (Figure 4C; supplemental Figure IV) induced by H_2O_2, suggesting that endogenous Ang II also interacts with H_2O_2 to elevate oxidative stress levels.

ACE activity and the expression of AT1 receptor mRNA in cultured ECs were determined. ACE activity calibrated by the protein concentration was not changed after H_2O_2 treatment: 106±9% at 3 hours and 103±8% at 24 hours after H_2O_2 treatment compared with the values at baseline and 3 hours after vehicle treatment (100±3% and 96±13%, respectively; n=3). The relative amount of the AT1 receptor to the housekeeping gene G3PDH, as measured by real-time PCR analysis, was not significantly changed after H_2O_2 treatment: 91±2% at 1.5 hours during the treatment, 99±5% at 3 hours, and 102±4% at 6 hours after H_2O_2 treatment compared with vehicle treatment (100±6%; n=3). Considering negative regulation in vascular smooth muscle cells[27,28] together, upregulation of the AT1 receptor is not likely to occur in response to H_2O_2 treatment.

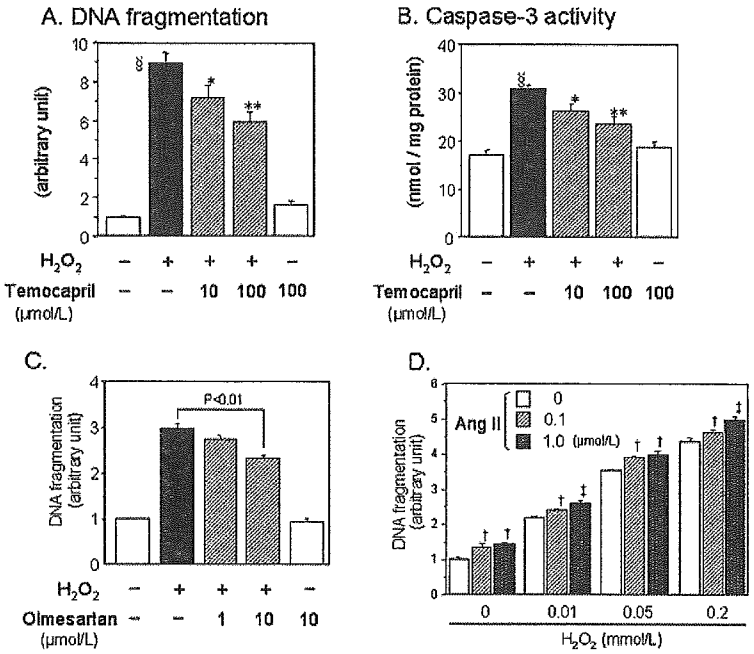

Figure 3. Effects of temocapril (A and B), olmesartan (C), and Ang II (D) on H_2O_2-induced EC apoptosis in culture. A through D, Temocapril, olmesartan, Ang II, or their vehicle was added to the culture medium 24 hours before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 hours after H_2O_2 treatment (0.2 mmol/L in A through C; 0.01 to 0.2 mmol/L in D) by means of DNA fragmentation (A, C, and D; n=3) and caspase-3 activity (B; n=4). §$P<0.01$ vs H_2O_2 (−). *$P<0.05$; **$P<0.01$ vs H_2O_2 (+) + temocapril (−). †$P<0.05$ vs Ang II (−). ‡$P<0.05$ vs Ang II 0.1 μmol/L. Values are expressed as mean±SEM. Similar results were obtained in 3 independent experiments.

Discussion

This study was conducted to elucidate the role of the RAS in oxidative stress–induced EC apoptosis using a rat model and cultured ECs. Treatment with H_2O_2 did not increase ACE activity or Ang II in the rat carotid artery during the acute phase. However, administration of an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, inhibited EC apoptosis in vivo. Furthermore, we demonstrated using cultured ECs that combination of Ang II and H_2O_2 dose-dependently increased EC apoptosis and 8-isoprostane formation. In addition, temocapril and olmesartan reduced but not canceled EC apoptosis and 8-isoprostane formation induced by H_2O_2, suggesting that endogenous Ang II interacts with H_2O_2 to elevate oxidative stress levels and EC apoptosis.

In vascular lesions such as atherosclerosis and intimal hyperplasia, the production of reactive oxygen species[4,5] as well as the components of the RAS[9–12] are upregulated, suggesting a possible interaction between them. A number of investigations have clarified that Ang II induces oxidative stress in vascular cells. Ang II stimulates the production of reactive oxygen species in ECs by upregulating the subunits of NAD(P)H oxidase: gp91 phox[17] and p47 phox.[18] It has been reported that the RAS enhances EC apoptosis in vitro[20,21] and contributes to endothelial dysfunction in patients with renovascular hypertension through the oxidant-dependent mechanism.[19] Conversely, it remains unknown whether oxidative stress could regulate the RAS; only 1 report has shown the modulation of ACE by oxidative stress.[29] Usui et al[29] reported that the inhibition of NO synthesis by chronic administration of L-NAME in rats augmented superoxide production and ACE activity in aortic ECs, and these effects were eliminated by treatment with

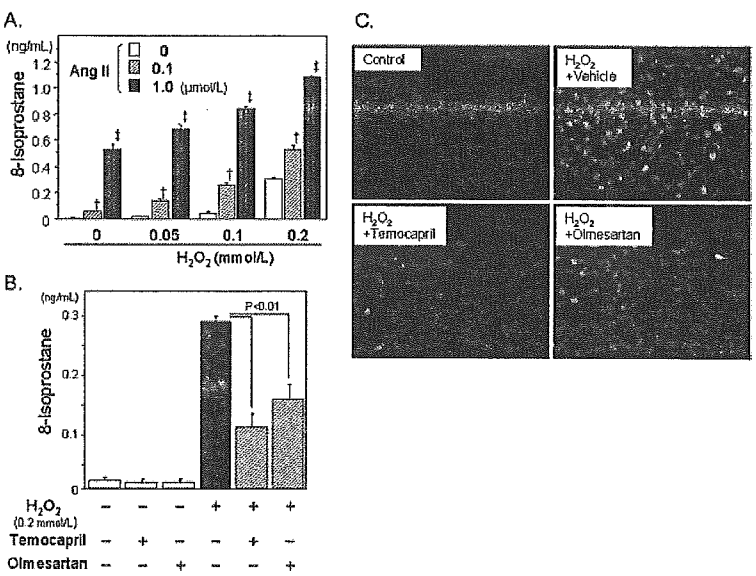

Figure 4. Effects of Ang II (A), temocapril, and olmesartan (B and C) on 8-isoprostane and DCF formation in cultured ECs. Ang II, temocapril (100 μmol/L), olmesartan (10 μmol/L), or their vehicle was added to the culture medium 24 hours before H_2O_2 treatment until assay. Then 8-isoprostane concentration in the culture supernatant and intracellular DCF intensity were measured 3 hours after H_2O_2 treatment. †$P<0.05$ vs Ang II (−). ‡$P<0.05$ vs Ang II 0.1 μmol/L. Values are expressed as mean±SEM (n=3). Similar results were obtained in 3 independent experiments.

antioxidants. In the present study, ACE activity in the carotid artery was not increased until 24 hours after H_2O_2 treatment. We also found that ACE activity was not changed after H_2O_2 treatment in cell culture experiments. Furthermore, the expression of AT1 receptor mRNA in cultured ECs, as measured using real-time PCR, was not increased after H_2O_2 treatment. Together, it is not likely that Ang II production or its receptor expression was upregulated in response to H_2O_2.

However, an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, inhibited H_2O_2-induced EC apoptosis in rats as well as in cell culture experiments. No influence of L-NAME on the antiapoptotic effect of temocapril in cell culture studies indicates that the effect of temocapril was attributable to the inhibition of Ang II synthesis. An AT2 receptor blocker, PD123319, did not influence H_2O_2-induced EC apoptosis either. This result appears to be inconsistent with the previous finding[30] but suggests a minimal contribution of the AT2 receptor in H_2O_2-induced EC apoptosis or minimal expression of the AT2 receptor in the cultured ECs used in the present study. Reduction in 8-isoprostane formation by temocapril and olmesartan suggests that endogenous Ang II adds to the oxidative stress levels on top of exogenous H_2O_2; otherwise temocapril and olmesartan would have antioxidant effects independent of Ang II through currently unknown mechanisms, although the in vivo role of bradykinin/NO in the effect of ACE inhibitors and that of the AT2 receptor remain to be addressed.

Administration of Ang II provided evidence that Ang II can interact with H_2O_2 to elevate oxidative stress levels and induce EC apoptosis. In rat experiments, a high and pressor dose of Ang II was used in combination with hydralazine[31] because 3-day administration of lower doses of Ang II (0.1 to 0.2 mg/kg per day) did not show significant effects on EC apoptosis (data not shown). The cell culture experiments to examine the effect of submaximal doses of Ang II and H_2O_2 on apoptosis and 8-isoprostane formation gave us clear information that AT1 receptor signaling augments EC apoptosis by an interaction with oxidative stress. Although the doses of H_2O_2 and the time duration of exposure were optimized on the basis of the time- and dose-response experiments, the conditions in cell culture studies were different from those in animal studies. However, it has been reported that cigarette smoke, oxidized lipoproteins, and polymorphonuclear leukocytes, which play important roles in atherogenesis, can generate H_2O_2 concentrations of 0.05 to 0.2 mmol/L in vitro.[32] These reports suggest that the dosages of H_2O_2 used in the present study do not far exceed the physiological range, although direct comparison of physiological or pathophysiological conditions with those in our experiments may be inappropriate.

Considering the stimulatory effect of Ang II on free radical production,[17–19] our finding that endogenous Ang II exacerbates EC apoptosis induced by exogenous H_2O_2 is not surprising. In fact, a number of reports have shown experimentally that RAS inhibitors can reduce the production of reactive oxygen species in pathological conditions such as peripheral arteries in rats with chronic heart failure,[33] rat diabetic nephropathy,[34] and kidney mitochondria in aged rats.[35] In the clinical setting, it is reported that administration of an AT1 receptor blocker (losartan) to patients with chronic renal disease reduced urinary excretion of oxidized albumin and malondialdehyde.[36] Also, 4-week treatment with losartan or an ACE inhibitor (ramipril) in patients with coronary artery disease diminished the response of endothelium-dependent vasodilation to intracoronary administration of antioxidant vitamin C in parallel with improvement of basal endothelium-dependent vasodilation,[37] indicating that RAS inhibitors can improve endothelial function in association with a reduction of oxidative stress. In the present study, we investigated EC apoptosis, an important process that leads to endothelial dysfunction and atherosclerosis[22,23] using an in vivo model. Moreover, our finding that RAS inhibitors attenuated EC apoptosis suggests broad end-organ protective effects of RAS inhibitors, which have been used for the treatment of hypertension and heart failure.

Perspectives

We found using an in vivo model and cultured ECs that Ang II elevated oxidative stress levels and increased EC apoptosis, whereas RAS inhibitors restrained them. These findings will add new information for cardiovascular research and the clinical application of RAS inhibitors.

Acknowledgments

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At baseline, the subjects showed moderate cognitive impairment and dependency and relatively low sex hormone levels (Table 1). After 3 months of exercise, significant increases were found in plasma levels of testosterone of 18%, estradiol of 38%, and DHEA of 37%, all of which returned to the baseline levels 3 months after cessation of the exercise program. A similar alteration was found in plasma DHEA sulfate level, but the increase by exercise was not statistically significant (mean ± standard error 452 ± 62 ng/mL at baseline, 508 ± 72 ng/mL after exercise, and 464 ± 77 ng/mL after discontinuation). Sex hormone–binding globulin, albumin, and other blood parameters did not change throughout the study (Table 1 and data not shown). Despite the increases in sex hormones after the exercise program, neither Barthel Index nor Mini-Mental State Examination scores changed significantly during the study.

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Measurement	Baseline	Exercise (3 Months)	Discontinuation (3 Months)
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Sex hormone–binding globulin, nmol/L	75.0 ± 6.1	69.1 ± 8.1	68.3 ± 8.3
Barthel Index	75.0 ± 5.4	70.0 ± 7.1	66.5 ± 9.4
Mini-Mental State Examination score	13.9 ± 1.9	13.8 ± 2.0	12.4 ± 2.5

$P <$ *.05; †.01 versus baseline using paired t test.

Previous studies[4,5] have shown stimulatory effects of endurance or resistance exercise on circulating hormones in healthy postmenopausal women; metabolic alterations and increased blood flow of endocrine organs via nitric oxide and cyclic adenosine monophosphate production may play a causal role, but hormonal responses in frail or demented women have not been examined. In the present study, plasma levels of estradiol, testosterone, and DHEA were higher after 3 months of physical exercise in elderly women with dementia, whereas cognitive function and basic ADLs did not improve. Given the protective effect of exercise and sex hormones on cognitive impairment, a control sedentary group should be included to examine whether this exercise program might delay cognitive decline. Nevertheless, the finding that exercise can increase plasma sex hormone levels in demented women provides a mechanistic insight into the effect of exercise or physical activities on cognitive impairment. The results of this preliminary study need to be confirmed using larger randomized, controlled trials with longer follow-up periods.

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Underdiagnosis of low-grade pressure ulcers in racial minorities, with subsequent progression to open lesions of higher grade, is largely consistent with these findings, as well as the findings of another study.[1] It has been long noted that the definition of low-grade pressure ulcers (persistent nonblanchable erythema) could result in the underdiagnosis of these lesions on dark skin. Because detection of low-grade pressure ulcers is an important factor in preventing their progression to higher stages, underdiagnosis likely contributes to the higher rates of high-grade ulcers found in older blacks and Hispanics. The relationship between contextual factors (such as resources and staffing) related to the types of nursing homes serving predominantly people of color and the underdiagnosis of pressure ulcers needs to be explored. Facilities serving primarily African Americans may have fewer funds and consequently offer fewer services and provide less staff training and amenities than other facilities.[2,3]

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ORIGINAL ARTICLE

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Estimation of Contrast of Refraction Contrast Imaging Compared with Absorption Imaging—Basic Approach

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Purpose: We discuss the usefulness of the refraction contrast method using highly parallel X-rays as a new approach to minute lung cancer detection. The advantages of refraction contrast images are discussed in terms of contrast, and a comparison is made with absorption images.
Materials and Methods: We simulated refraction contrast imaging using globules with the density of water in air as models for minute lung cancer detection. The contrast intensified by bright and dark lines was compared on a globule with the contrast of absorption images. We adopted the Monte Carlo simulation to determine the strength of the profile curve of the photon counts at the detector.
Results: The obtained contrasts were more intense by two to three digits than those obtainable with the absorption contrast imaging method.
Conclusion: The contrast in refraction contrast imaging was more intense than that obtainable with absorption contrast imaging. A two to three digit improvement in contrast means that it is possible to greatly reduce the exposure dose necessary for imaging. Therefore, it is expected to become possible to detect the interfaces of soft tissues, which are difficult to capture with conventional absorption imaging, at low dosages and high resolution.

Key words: synchrotron radiation, refraction contrast, ray-tracing method, surface dose

Introduction

Remarkable progress has been made in X-ray imaging diagnostic equipment technology. However, conventional absorption imaging has the limitation that its application to the observation of objects with small absorption rate differences is difficult. Expectation is mounting for the development and realization of a next-generation coherent X-ray source similar to that of the laser. Refraction contrast by X-ray (synchrotron radiation) of such a high degree of coherence enables higher contrast imaging, which reflects the object density difference, than that of the absorption imaging method.[1] Thus, it has possibilities for clinical application in the future.[2] Refraction contrast imaging by generating bright and dark lines on the object interface[3] has the effect of improving visibility.[4,5] It was impossible to obtain such images with conventional radiation sources due to their low degree of coherence. However, third-generation radiation sources have enabled highly coherent imaging. The contrast imaging can be applied to the fields of orthopedics, breast cancer, and respiratory systems, among others. In this study, we applied X-ray tracing using Snell's law to the determination of contrast provided by the refraction contrast method and made comparisons with contrast

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provided by the absorption method. A study has demonstrated that it is possible to obtain high resolution images of lung tissue from excised samples by using refraction contrast imaging by synchrotron radiation.[5] In this study, we examined whether it is possible to obtain high resolution images of live human lung cancer by using this method. For simplification, we used a model that assumes the existence of globules with a density equal to water for evaluation of the method.

Materials and Methods

Lung cancer is known to emerge in the central or peripheral regions, while the regions around tumors are surrounded by air. This paper assumes the existence of globules with an equal density to water in air for simplification of the X-ray imaging simulation.

The refraction index for X-rays is usually expressed $n=1-\delta+i\beta$. Both δ and β are very small. For example, in water, δ is 2.3×10^{-7} and β is 1.2×10^{-10} at 30 keV. Therefore, the refraction index is much larger than the index of absorption β. The real part of the refractive index is smaller than 1.0 but close to 1.0, so the refraction angle is small. In Fig. 1, the refraction angle is $\pi/4 + 0.23\times10^{-6}$ rad (incident angle $\pi/4$ rad).

Parallel monochrome X-rays were illuminated to objects assumed to be soft tissues with diameters of 24 to 1,000 μm and a density of 1.0 in order to obtain their images 0.25 to 10 m behind the samples (refraction) and immediately posterior to them (absorption). The Monte Carlo simulation was applied to the calculation of the travel paths of X-rays along the object surfaces, and absorption is considered in this paper. The number of photons radiated into the pixels of the detector was counted, and the number of counts was interpreted as intensity. The simulation was performed with an energy level of 5 to 60 keV. Thus, determining the density of the globular object, the diameter of the globule, X-ray energy, and the distance between the object and the detector (sample distance) makes it possible to apply the ray-tracing method to the calculation of contrast: C=(Imax–Imin)/(Imax+Imin), (Imax: maximum value of intensity, Imin: minimum value of intensity).

Results

1. Advantages of refraction contrast images over absorption images in terms of contrast

Given that pixel size is 6 μm when the object is in air, bright and dark lines caused by refraction appear in refraction contrast images as explained in Materials and Methods, resulting in contrast intensification (Fig. 1). First, the accuracy of this simulation is tested with

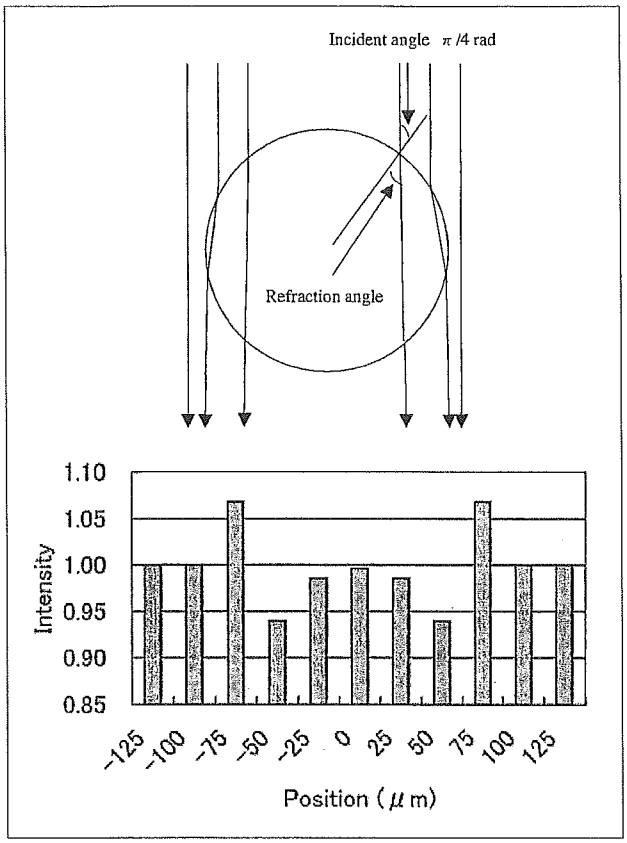
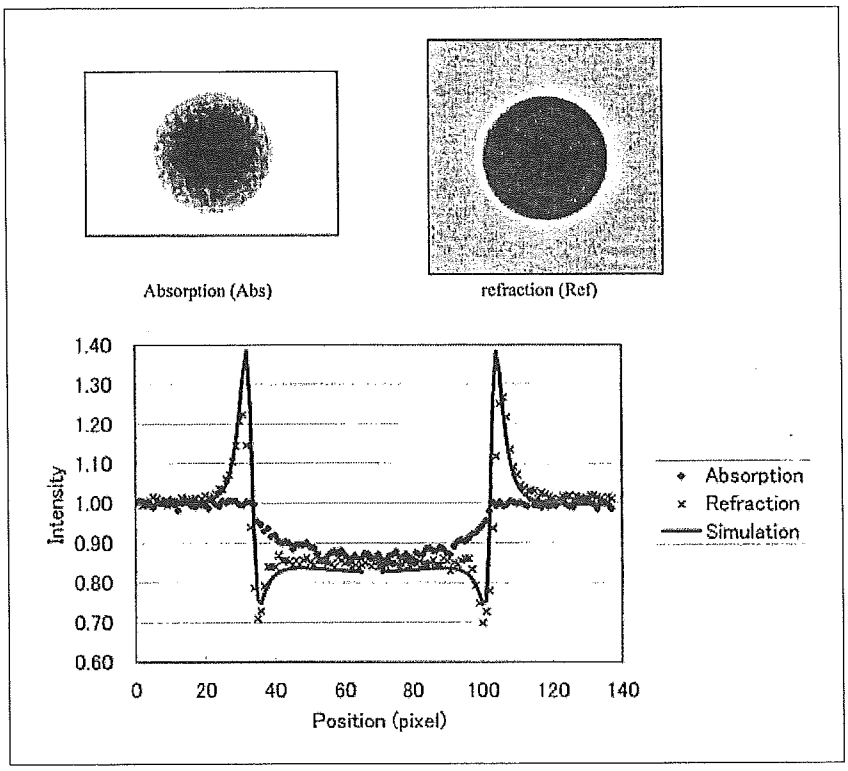


Fig. 1A. Refraction contrast imaging.
The parallel X-ray beams are bent by refraction, generating bright and dark lines outside and inside the boundary lines, respectively.

A
B

Fig. 1B. Profile curve of 30 keV X-ray refraction by a globule with a diameter of 100 μm (specific density = 1.0) observed with a detector with a pixel size of 25 μm (the background is normalized to 1.0).

experimental data. The orbit of X-ray photon is calculated using the density and absorption coefficient of the object and surrounding object in this simulation. A sapphire with a diameter of 400 μm in air was scanned with a CCD camera with a pixel size of 6 μm, applying 24.8 keV monochromatic X-rays at BL-28 in SPring-8. The images and profile curves (Fig. 2), obtained at a sample distance of 175 cm with a scanning duration of 6 seconds, were observed to correspond well to the results of calculation by the ray-tracing method. As for contrast, the obtained experimental value was (1.27–0.70)/(1.27+0.70) = 0.29, which corresponds closely to the simulated contrast value of 0.30. For the absorption image, the sample distance was set at 1 cm, and the scanning duration at 6 seconds. Table 1 shows the contrast measured with an absorption image and that obtained in refraction contrast imaging. The refraction image of a 2 mm ball pen refill is shown in Fig. 3B for



A

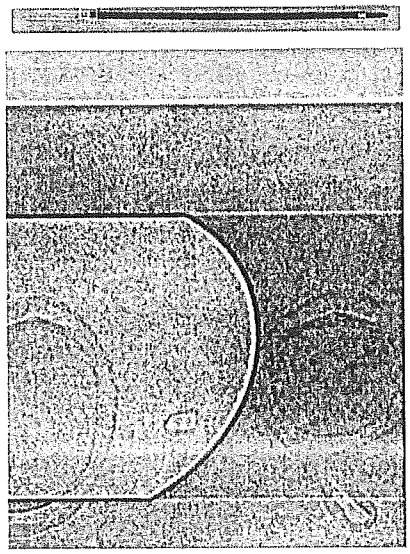
B

Fig. 2A. Absorption image and refraction contrast image of a sapphire globule with a diameter of 400 μm at sample distances of 1 cm (abs) and 175 cm (ref).
Fig. 2B. Experimental and calculated data at 24.8 keV, duration 6 seconds.
(1) At a sample distance of 175 cm (red square dots), refraction contrast is observed to be intense.
(2) At a sample distance of 1 cm (blue square dots), the graph is considered to be almost equivalent to the curve of the absorption images.

Table 1. Comparison of contrast of sapphire globule according to imaging method (data obtained by experiment)

Imaging method	Contrast
Absorption	0.07
Refraction	0.29

Table 1 shows that the contrast in refraction contrast images is more intense than that in absorption images. The experiment shows that better contrast is obtainable in refraction contrast images than in absorption images. Figure 2 shows the images referred to in Table 1 and the profile curves along the line through the center.



A

B

Fig. 3A. Reference optical image (ballpoint pen refill with a diameter of 2 mm).
Fig. 3B. Reference refraction contrast image (ballpoint pen refill with a diameter of 2 mm) at 33 keV.

reference. The absorption at the center of the ball pen refill is approximately 0.05, but the ball pen is clearly recognized. Even printed figures are also clearly recognized.

Figure 4 shows the results of the calculation of the contrasts obtained in refraction contrast imaging and absorption imaging with sample size as the parameter. The energy is 30 keV. The contrast in absorption images decreases almost in proportion to the size of the sample to be observed. Meanwhile, the decrease in the contrast of refraction images is relatively small. Therefore, when the sample size is small, refraction contrast is more advantageous in terms of contrast. Figure 4 also shows the images of globules with diameters of 1 mm, 100 μm, and 24 μm to demonstrate the relative advantage of refraction contrast imaging over absorption imaging in terms of contrast. The contrast in the refraction contrast image is 19 times greater than that in the absorption image even when the globular object has a diameter of 1 mm and 255 times greater and more advantageous by two digits when the diameter of the globular object is 24 μm.

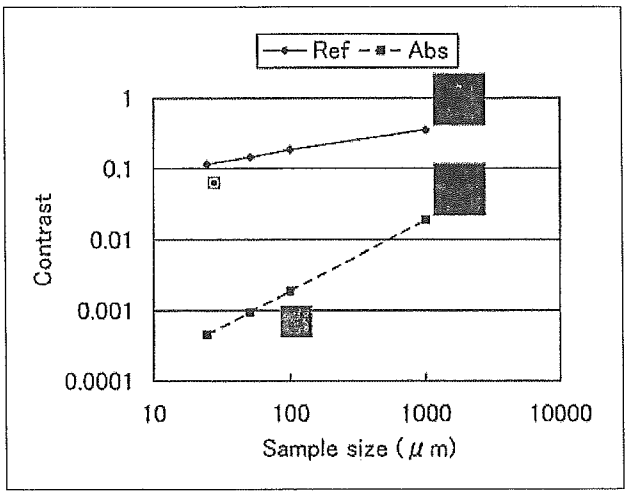


Fig. 4. Sample size dependence of refraction contrast compared with absorption imaging.
The contrast in refraction contrast images is more intense by about two digits than that in absorption images when the conditions of distance between the sample and detector (hereafter referred to as sample distance) are 1 m, X-ray energy is 30 keV, and detector pixel size is 6 μm. X-ray images and their profile curves of intensity of globules 1 mm, 100 μm, and 24 μm are shown. Contrast by the refraction contrast method does not decrease remarkably in relation to sample size reduction. Meanwhile, contrast by the absorption method decreases in reverse proportion to sample size. Therefore, when the size of the observed object is small, higher visibility is achievable with the refraction method. The contrast of a globule with a size of 1 mm is slightly recognizable with the absorption method, and no image can be obtained at all with a globule of smaller size.

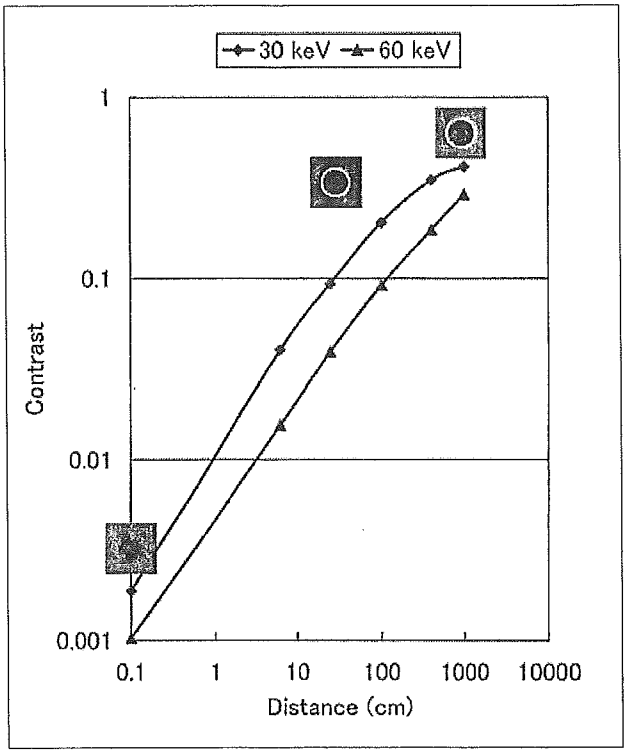


Fig. 5. Sample distance dependence of refraction contrast compared with absorption imaging.
The contrast intensifies in response to the increase in distance with a sample size of 100 μm and detector pixel size of 6 μm. X-ray images of sample distances of 10 m, 4 m, 1 m, 25 cm, 6.25 cm, and 100 μm (absorption image) and their profile curves of intensity are shown. The contrast of the absorption images remains constant independently of the sample distance, and its intensity is between the 15.1 and 282 of that obtained in the refraction images.

2. Intensification of contrast in proportion to sample distance and fading in reverse proportion to energy

(1) Sample distance and contrast

Figure 5 shows the relation between the contrast and sample distance using X-ray energy as a parameter. Figure 5 also shows the images of a 100 μm globule obtained when sample distance is changed from 100 μm (absorption) to 10 m as parameters. Intensity profile curves on a line across the image center are shown next to each image. The contrast is observed to intensify when the increase in sample distance causes bright and dark lines to appear outside and inside the outline of the globule, respectively. The contrast remains constant at 0.0037 for absorption images (distance 100 μm), as shown in Fig. 5.

Figure 5 shows the dependence of contrast on distance to demonstrate that contrast intensifies in response to an increase in distance.

(2) Contrast intensification dependent on energy decrease

Figure 6 shows contrast intensification depending on the decrease in energy from 60 keV to 30 keV and 10 keV to observe a globule with a diameter of 100 μm. The dependence of contrast on energy is shown with the sample distance at 2 m, 1 m, and 25 cm. The contrast fades according to the increase in energy. The longer sample distance along with the lower energy intensifies the contrast. However, the lower energy intensifies the contrast by absorption, diminishing the advantage of refraction.

3. Change in contrast according to globule-detecting resolution in air and sample size

(1) Contrast intensification in proportion to resolution of the detector

Figure 7 shows how the contrast in the image of a globule with a diameter of 100 μm intensifies according to the reduction in pixel size from 25 to 6 to 1 μm. Figure 7 also shows an image and profile curve for each pixel size, and the relation between contrast and pixel size

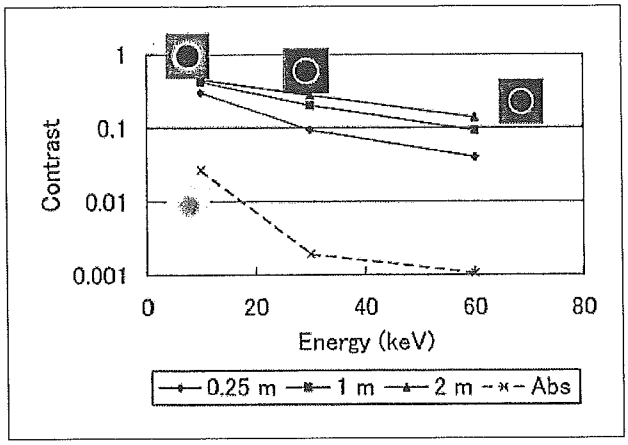


Fig. 6. Energy and sample distance dependence of refraction contrast compared with absorption imaging.
X-ray images of sample distance 1 m, 100 μm (absorption image) and their profile curves of intensity are shown. Figure 6 shows that, with sample distance constant, energy reduction improves contrast. This effect is enhanced as sample distance increases.

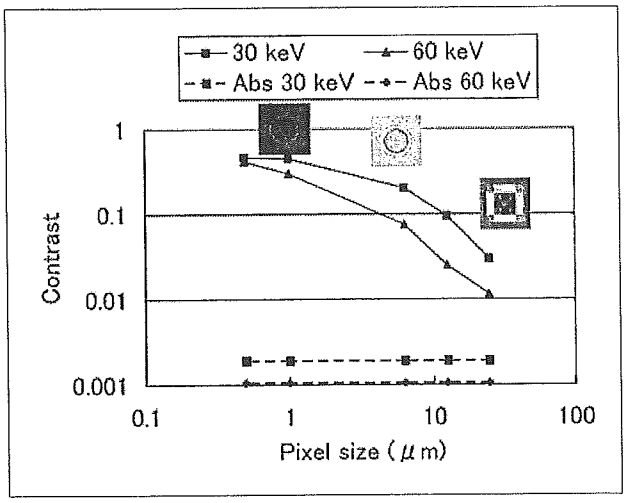


Fig. 7. Detector pixel size dependence on refraction contrast. X-ray images of pixel size of 25 μm, 6 μm, and 1 μm and their profile curves of intensity are shown. These images show that the reduction in pixel size intensifies the contrast.

(including pixel size 0.5 μm). While the number of photons necessary to measure refraction contrast images increases in inverse proportion to detector pixel size Px, the reduction in pixel size makes the difference between bright and dark lines remarkable, resulting in the intensification of contrast. Therefore, the total number of necessary photons increases only moderately. Note, however, that the decline in detector efficiency is ignored here. Noise has an important role for contrast in finite photon numbers. However, because the aim of this study was to estimate contrast, noise is ignored here. To exploit the advantages of the refraction contrast method, it is necessary to reduce the pixel size of the detector.

As can be seen in the images shown in Fig. 7, the refraction effect makes the difference between bright and dark lines remarkable in response to the reduction in pixel size. Because of this, contrast is also intensified, as shown in the profile curve (30 keV, sample distance fixed constant at 1m).

(2) Invariance of contrast against sample size reduction

Figure 8 shows the change in contrast in response to the reduction in sample size for each detector pixel size at an energy level of 30 keV. We have already shown in Result 1 that contrast remains constant against the reduction in sample size. It is shown here that refraction contrast is more advantageous than absorption contrast to observe minute objects.

4. Dependence of the contrast on difference in density

Figure 9A and 9B show images of 100 μm globules with specific gravities of 1.1, 1.01, and 1.001 placed as

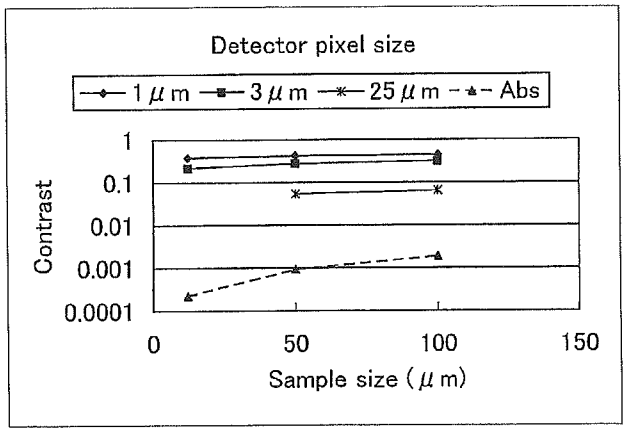


Fig. 8. Sample size and pixel size dependence on refraction contrast.

in Fig. 10 in the center of a water layer 200 μm thick to study the differences in refractive index. Figure 9A and 9B also shows the dependence of contrast on pixel size and density ratio, which are also shown in Tables 1 and 2. The X-ray energy is set at 60 keV and 5 keV. It is advantageous in terms of contrast to keep energy low and pixel size small, because it is difficult to detect refraction between small density differences in high enregy.

The tables suggest that the contrast-intensifying effect caused by refraction is strong where a large density difference is present, while the effect diminishes with a density difference as small as 0.1%. Since the reduction in detector pixel size to 6 μm makes it possible to obtain contrast of 0.04 at 5 keV, even where a density difference

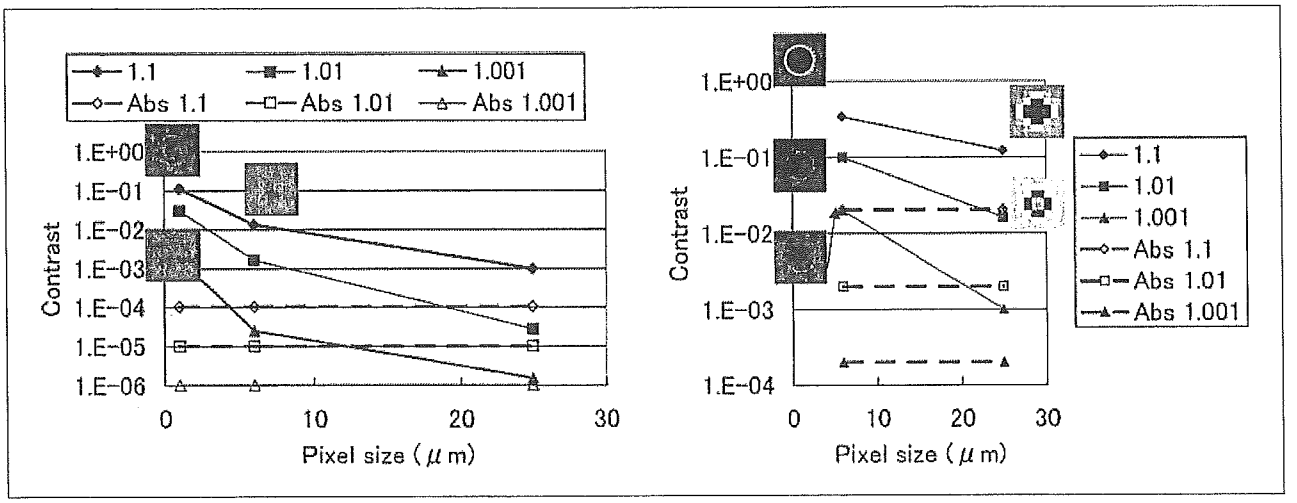


Fig. 9A. Relation between contrast and pixel size with various density differences at an X-ray energy level of 60 keV.
(1) Images are hard to visualize by absorption at 60 keV with the density difference at 1.1, 1.01, or 1.001.
(2) No good contrast is obtainable with a pixel size of 25 μm, thus making visualization by refraction difficult, too. Even with a pixel size of 6 μm, visualization is possible only when the density difference is 1.1. When pixel size is 1 μm, contrast remains at 0.031 down to a density difference of 1.01, thereby making visualization somehow possible.
Fig. 9B. Relation between contrast and pixel size with various density differences at an X-ray energy level of 5 keV.
(1) The contrast in absorption images is 0.04 at 5 keV when the density difference is 1.1, making visualization possible. When the density difference is 1.01 or 1.001, the contrast is 0.002 or 0.0002, making visualization difficult.
(2) A contrast of 0.12 or 0.017 is obtained by refraction with pixel size at 25 μm when the density difference is 1.1 or 1.01. Thus, visualization by refraction becomes possible as shown above. With the density difference at 1.001, the obtained contrast is 0.001, making visualization difficult. With the density difference at 6 μm, the obtained contrast is 0.02 even at a density difference of 1.01, making visualization possible. With the density difference at 1 μm, the obtained contrast is 0.16, resulting in a good image. Table 2 summarizes visualization performance.

as small as 0.1 is present, the contrast is still considered to be measurable. Moreover, with the pixel size at 1 μm, it is possible to obtain sufficient contrast of 0.031.

To intensify contrast by the refraction contrast method in relation to density difference, it is necessary to reduce the pixel size of the detector. Contrast intensifies in inverse proportion to pixel size. Yet, with the density difference at 1.001, the contrast obtainable with a pixel size of 1 μm is as small as 0.0035. It takes a density difference of at least 1.01 to visualize the border (Fig. 9A).

In this case, even with a small density difference, better contrast is obtained than that obtainable at 60 keV. The reduction in pixel size to as small as 6 μm improves contrast by two digits, making it 100 times more intense than that obtainable by absorption (Fig. 9B).

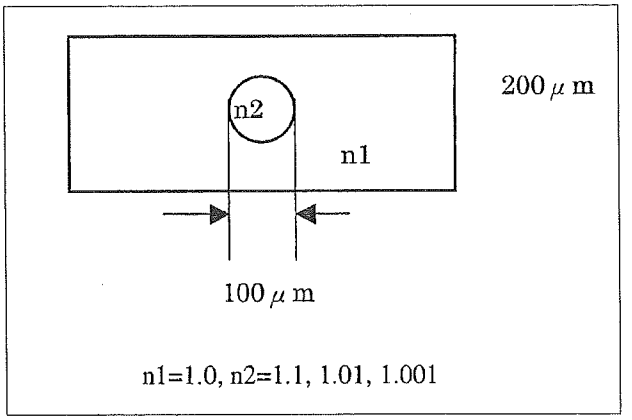


Fig. 10. Schematic diagram of a tabular region 200 μm thick with a density of 1.0 containing a globular region with a diameter of 100 μm (density n2: 1.1, 1.01, and 1.001) at its center.
See Figs. 9A and 9B for images of the globular region shown in Fig. 10 by the absorption method and the refraction contrast method, respectively.

DISCUSSION

In this study, we examined the contrast ratio in refraction contrast imaging and absorption contrast imaging. According to Reference 6, when the absorption method is applied, the number of necessary photons changes in proportion to X^{-4}, where X denotes the size of the object to be identified. The number of necessary photons increases to detect smaller obstacles. Thus, in the absorption method, the dose reaches as high as 160 Gy at 30 keV when detecting a globule with a diameter of

Table 2. Contrast at 60 keV, sample size 100 μm

Density	Pixel size	Contrast by absorption	Contrast by refraction	Ref/abs
1.1	25	0.00011	0.001	9.5
	6		0.014	129
	1		0.11	1048
1.01	25	1.0E-05	2.8E-5	2.7
	6		0.0017	160
	1		0.031	2961
1.001	25	1.0E-06	1.6E-6	1.5
	6		2.5E-5	24
	1		0.0018	1699

Table 3. Contrast at 5 keV

Density	Pixel size	Contrast by absorption	Contrast by refraction	Ref/abs
1.1	25	0.02 (>)	0.12 (>)	6
	6		0.34 (>)	17
	1		0.46 (>)	23
1.01	25	2.0E-03 (<)	1.6E-2 (>)	8
	6		0.10 (>)	50
	1		0.40 (>)	198
1.001	25	2.0E-04 (<)	0.001 (<)	5
	6		0.020 (>)	100
	1		0.016 (>)	775

Visualization performance is indicated in parentheses: (>) means a contrast of 0.01 and over, while (<) means contrast below that. Sample size = 100 μm.

100 μm with detector pixel size at 25 μm. Meanwhile, the contrast provided by the refraction contrast method is 19 times more intense than that obtainable with the absorption imaging method, and, with the pixel size at 6 μm, 105 times more intense, making detection even more advantageous. Further reduction in sample size to 50 μm and then to 24 μm diminishes the contrast obtained by absorption to 1.88×10^{-3} and 9.01×10^{-4}, respectively, while the contrast provided by refraction remains almost constant.

In absorption imaging, given that the S/N ratio at which objects of the same size are detected is kept constant, the necessary dose (number of photons) is reduced at least in proportion to Px^{-2}, where Px denotes pixel size. Conversely, since the contrast between refracted bright and dark lines intensifies in response to pixel size reduction in refraction imaging, the necessary dose is not reduced in proportion to Px^{-2}. The smaller the pixel size, the sharper the image, resulting in more intense contrast (see Fig. 7). In this respect, the refraction contrast imaging method is expected to be applied to next-generation X-ray imaging that has the advantages of high resolution and low exposure dosages. Refraction contrast imaging can demonstrate the effectiveness of its advantages in the X-ray energy range between 30 and 60 keV only when the material density difference of the interface is large. Therefore, refraction contrast is effective for the detection of locations where a large material density difference is present, such as the interface between air and biological materials or that between bones and biological materials.

However, if a detector pixel size as small as 1 μm were available at energy as low as 5 keV, good refraction contrast could be obtained even at a density difference as small as 0.1%. Although no detectors with a pixel size of 1 μm exist in reality, it would be highly useful if they did. Realistically, it might be possible to realize the advantages of such a pixel size by using films with a resolution of 1 μm. Still, the application of a low X-ray energy of 5 keV to the human body would be impractical. Moreover, the effects of multiple scattering have not been quantitatively dealt with in past discussions. Since achievable refraction contrast is expected to be lower than that obtained by calculation because of scattered

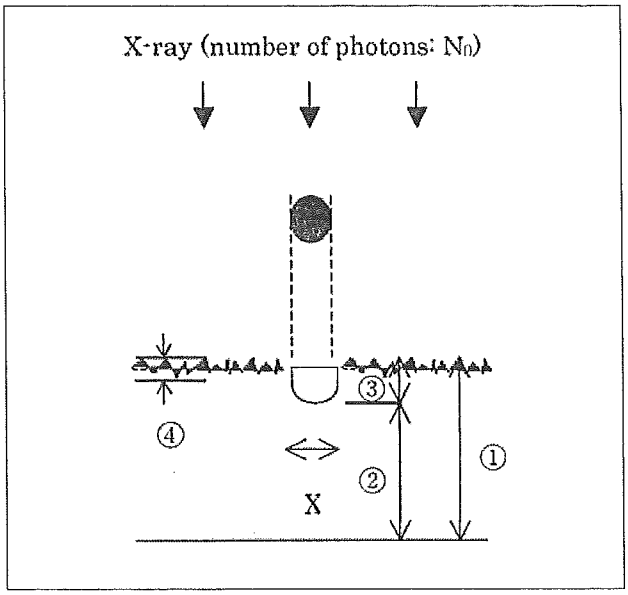


Fig. 11. Absorption imaging: schematic diagram of parallel X-ray irradiation on a globule.

radiation, experimental verification is needed.

In addition, it would be necessary to take into consideration that high X-ray energy results in low efficiencies of detectors. In Fig. 11, Item ① is the number of X-ray photons when no object is present. X indicates the diameter of an object. Item ③ is the reduction in the number of photons due to absorption by the object, Item ② indicates the number of photons penetrating the object, and Item ④ indicates the variation of the background. Given that the water attenuation coefficient in Fig. 11 is μ, Item ③, is expressed by $k = N_0\{1-\exp(-\mu X)\}/N_0$.

In the case of refraction contrast imaging, given that the peak intensity of a bright line is N (bright), the lowest intensity of a dark line is N (dark), and the number of photons in the background is N_0, k is defined as {N (bright) – N (dark)}/N_0.

Here, let R be efficiency, then the required dose (number of photons) is expressed by N_0 (min.) = $8/(Rk^2)$ in Reference 6, and the dose becomes effective both in absorption and refraction with the same factor. The radiation dose of refraction contrast imaging decreases compared with those of absorption imaging with same efficiency of detector.

Another issue to be considered is the speckle effect caused by the overlapping of images of objects with long depths. This will be discussed in another paper.

In this paper, we discussed the contrast-intensifying effect of the refraction contrast method, based on calculations obtained by the ray-tracing method. Using experimental data for comparison, we have demonstrated that the ray-tracing method is sufficient to reproduce the contrast obtained by the refraction contrast method. We have shown that the advantage of refraction imaging becomes greater in terms of contrast ratio than that of conventional absorption imaging in inverse proportion to the size of the object and the pixel size of the detector. We have also shown that, where a small detector pixel size is available at low energy, good refraction contrast can be obtained even when the density difference is small. A two to three digit improvement in contrast means that it is possible to greatly reduce the exposure dose necessary for imaging. The refraction contrast method is expected to be applied to next-generation clinical X-ray imaging.

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