TABLE 1

*Risk of rupture associated with UCAs in Japan**

Category	No. of Included Studies	No. of Cases	Patient-Years	No. of Ruptures	% Risk of Rupture per 100 Patient-Years (95% CI)	RR (95% CI)	OR of Rupture (95% CI)
overall	13	922	3801	104	2.7 (2.2–3.3)		
patient sex	10						
male†		294	1370	30	2.2 (1.5–3.1)		
female		435	1803	55	3.0 (2.3–4.0)	1.4 (0.9–2.2)	1.3 (0.85–2.22)
patient age (yrs)	11						
<60†		297	1543	35	2.3 (1.6–3.2)		
≥60		520	2077	62	3.0 (2.3–3.8)	1.2 (0.8–1.9)	1.17 (0.74–4.96)
history of SAH	13						
no†		709	2786	62	2.7 (2.1–3.4)		
yes		209	1010	28	2.8 (1.8–4.0)	1.3 (0.85–2.0)	1.06 (0.64–1.74)
symptoms present	13						
no†		876	3657	94	2.6 (2.1–3.1)		
yes		42	137	10	7.3 (3.5–13.4)	2.1 (1.1–3.9)‡	2.20 (0.97–4.96)
aneurysm site	10						
ant†		770	3370	59	1.8 (1.3–2.3)		
pst		127	618	20	3.2 (2.0–5.0)	2.3 (1.4–3.7)‡	2.18 (1.23–3.86)‡
aneurysm size (mm)	11						
<10†		585	2045	31	1.5 (1.0–2.2)		
≥10		100	344	32	9.3 (6.4–13.1)	6.4 (4.0–10.4)‡	8.68 (4.59–16.41)‡

* Ant = aneurysm located in the anterior circle of Willis; pst = aneurysm located in the posterior circle of Willis.
† Referenced category.
‡ Statistically significant RR or rupture rate and OR of rupture were obtained according to the method described by Breslow and Day. In the table the OR represents (odds of rupture in the group)/(OR of rupture of the referenced group).

been obliterated or no other known source of hemorrhage was detected. 5) The UCA was diagnosed using either conventional or digital subtraction angiography, magnetic resonance angiography, three-dimensional computerized tomographic angiography, or a combination of these methods. 6) Documentation of the rupture of the aneurysm (that is, the SAH) could be confirmed with the aid of computerized tomography scanning, lumbar puncture, surgery, or a postmortem examination.

If several publications originated at the same institution, the most recent series or the series including the largest number of cases was selected for review. In studies missing some inclusion criteria,[17,19,20,27,28] we sent requests to the authors and included the study if they could provide the missing data.

Data Extraction and Analysis

Once a study was deemed eligible for review, two of us independently extracted the following data: the total number of cases, the period of follow up, and the number of aneurysm ruptures. We also sent out requests to authors of the studies for additional detailed data not described in the papers. When data were available for stratification, we extracted information concerning patient age (11 studies) and sex (10 studies), the size of the aneurysm (11 studies), the site of the aneurysm (10 studies), any history of SAH (all 13 studies), symptoms caused by the aneurysm (all 13 studies), and incidences of death from SAH (11 studies). If data were available, we also extracted the number of surgeries performed on UCAs during the same period of study at the same institution, and the timing of lesion rupture. The influences of high blood pressure or smoking and their association with the rupture were discussed in very limited series[2,7,17] and were not assessed in this review.

To calculate the risk of aneurysm rupture, we multiplied the total number of patients by the average period of follow up in each study to obtain the total number of patient-years of follow up. The number of patients with subsequent SAH was then divided by the number of patient-years to yield the risk of rupture per 100 patient-years.[22] We used this method to calculate the risk of rupture in all patients and in the various subgroups. We asked the authors of all 13 studies to provide follow-up periods for each subgroup, but only four authors could do so.[17,26–28] Therefore, the other subgroups were analyzed using the average follow-up period of the total number of cases in each study.[3,22]

Analysis of the Review

To identify any variations among the studies in this review, we compared data between factors in the following subgroups: 1) studies including more than and fewer than 50 patients; 2) studies published in English and those in Japanese; and 3) studies from four districts: Touhoku, Kanto, Kinki-Hokuriku, and Chyuugoku-Shikoku.

Comparison With Large-Scale Cohorts and Other Retrospective Reviews

We compared the findings of our review with those of two large-scale international cohort studies (the ISUIA retrospective and prospective cohorts)[11,12] and a systematic review by Rinkel, et al.[22] The review of Rinkel and colleagues incorporated three Japanese series, and we analyzed the data both to include and exclude Japanese cases. We also compared patient characteristics and the overall rupture rate obtained by calculating total patient-years and total cases of rupture.

Statistical Analysis

The chi-square and Fisher exact tests were used to com-

TABLE 2

*Summary of included studies**

Authors & Year	No. of Cases	Patient-Years	No. of Ruptures	% Rupture Rate (95% CI)	No. of Deaths	Location of Institution
Inagawa, et al., 1992	47	240	1	4.0 (0–1.5)	NA	CS
Asari & Ohmoto, 1993	54	197	11	5.6 (2.8–10.0)	10	CS
Mizoi, et al., 1995	49	211	4	1.9 (0.5–4.9)	3	TH
Yasui, et al., 1997	234	1463	34	2.3 (1.6–3.2)	18	TH
Ikawa, et al., 1998†	36	155	7	4.5 (1.8–9.3)	7	CS
Yasui, et al., 1998†	10	25	1	4.0 (0–14.8)	0	KH
Ikeda, et al., 2000†	33	158	11	6.9 (3.5–12.4)	NA	KH
Tsutsumi, et al., 2000	62	267	7	2.6 (1.1–5.4)	6	TH
Murata, et al., 2001†	48	121	4	3.3 (0.9–8.4)	1	KH
Suga, et al., 2002†	100	317	5	1.6 (0.5–3.7)	3	CS
Tsukahara, et al., 2002	110	217	7	3.2 (1.3–6.6)	2	NA
Matsumoto E, et al., 2003	48	158	7	4.4 (1.8–9.1)	7	KT
Matsumoto K, et al., 2003	91	273	5	1.8 (0.6–4.3)	4	KH

* CS = Chyuugoku-Shikoku district; KH = Kinki-Hokuriku district; KT = Kanto district; NA = not available; TH = Touhoku district.
† Published in Japanese.

pare differences between risk factors and the studies. The Mantel–Haenzel method was also used to compare differences in the included series and to obtain RRs and ORs between subgroups.[3] A probability value less than 0.05 was considered significant. Statistical analyses were performed with the aid of a commercially available software program (SAS, version 8; SAS Institute, Inc., Cary, NC).

Results

Rupture Rate of Untreated UCAs in Japanese Institutions

Thirteen studies fulfilled our inclusion criteria.[2,6–8,16,17,19,20,24–28] Of these, eight were reported in English and the other five in Japanese. Twelve authors responded to our requests and nine provided additional information regarding subgroups.[6,8,17,19,20,24,26–28] Five studies included only asymptomatic cases,[7,20,24,26,28] and two studies included only cases without SAH.[24,26] One study included only aneurysms located in the anterior circulation,[17] and one included only patients older than 70 years.[28] We were able to incorporate these studies because the numbers of patients and ruptures were provided. A summary of the studies is shown in Table 1.

The 13 studies included 922 patients with UCAs who were followed up for a total of 3801 patient-years. There were 104 subsequent ruptures among the entire patient population (11%), constituting an annual rupture rate of 2.7% (95% CI 2.2–3.3%). The risk of rupture was significantly higher for large aneurysms (RR 6.4, 95% CI 4.0–10.4), aneurysms located in the posterior circulation (RR 2.3, 95% CI 1.4–3.7), and symptomatic aneurysms (RR 2.1, 95% CI 1.1–3.9) compared with each referenced category. Unruptured cerebral aneurysms tended to burst more often in patients presenting with SAH, in women, and in patients older than 60 years, but the differences between these groups were not significant. Because our rupture risks were calculated on the basis of an estimated average follow-up period in some series, we also obtained the OR for the net number of all cases of ruptured aneurysms/all cases of unruptured aneurysms in each subgroup, which would not be affected by the follow-up period. The OR of ruptured lesions in the subgroups also showed a similar tendency for a high rupture risk in large aneurysms, posterior-circulation lesions, and aneurysms with symptomatic presentation (ORs 8.68, 2.18, and 2.20, respectively).

In eight reports the authors discussed the risk of rupture of small aneurysms (< 5 mm); seven of 40 ruptured aneurysms in these studies were smaller than 5 mm.[6,8,17,19,20,24–26] Among patients with aneurysms measuring 5 or 6 mm, an additional four of 40 lesions ruptured; hence, 27.5% (11 aneurysms) of the ruptured aneurysms were smaller than 7 mm. Because of the low number of small aneurysms and the lack of details about the follow-up period, we could not calculate the rupture rate for this group.

Data on this incidence of death caused by SAH from 11 studies showed that 61 (66%) of 92 patients died of SAH.

Eleven reports provided data about surgery during the same period as the studies.[2,7,8,16,17,19,20,25–28] In these 11 studies, surgery was performed in 1601 patients, compared with 787 who were only observed.

The timing of rupture was documented in 11 series, but the total number of ruptures in each year could be obtained from only eight series.[2,6–8,20,24,26,28] Among 47 cases of subsequent rupture, 19 lesions ruptured within 1 year, nine ruptured within the 2nd year, and the remaining 19 aneurysms ruptured after the 2nd year. When we included three additional series that provided Kaplan–Meier life tables showing the timing of rupture,[16,17,27] we noted no acute increase in the rupture rate in any specific year.

Variations Among the Included Studies

A summary of the included studies appears in Table 2 and Fig. 1. The series reported by Ikeda and colleagues[7] and Inagawa, et al.,[8] demonstrated statistically significant differences when they were compared with other studies regarding the rupture rate ($p = 0.0068$). Excluding these two studies from the analysis did not alter the results of our review. Although the overall rupture rate was higher in studies written in Japanese and in those studies including fewer than 50 patients, no significant difference was noted between groups. Geographic location, which can influence the patient's living environment, did not influence the risk of rupture.

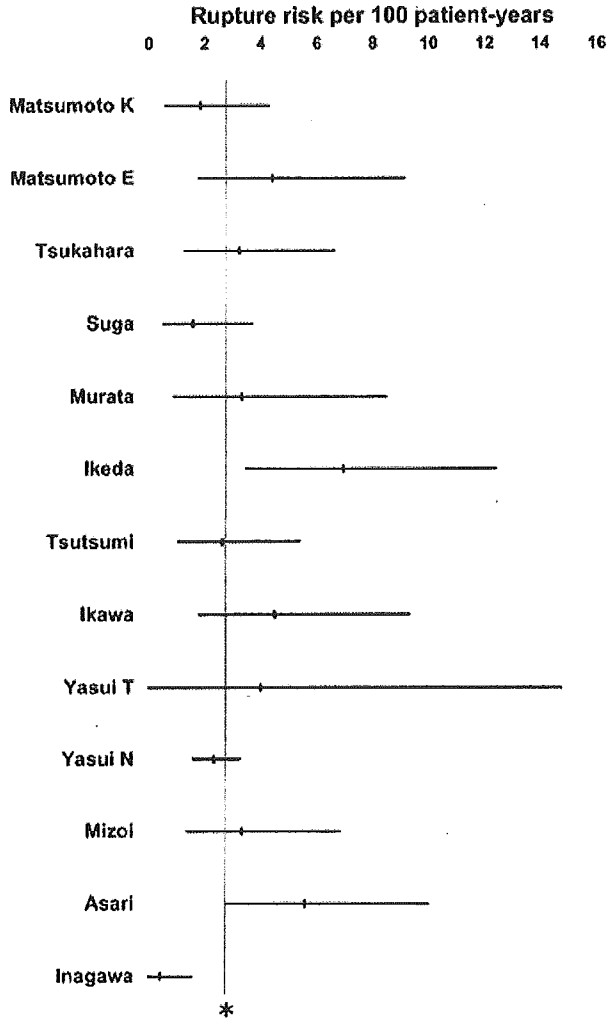

FIG. 1. Chart showing the risk of rupture associated with UCAs in the included studies. The *asterisked line* indicates the average rupture rate of all cases.

Comparison With International Studies

Table 3 offers a comparison of our findings and those of the ISUIA[11,12] and the systematic review from Europe by Rinkel and colleagues.[22] The ISUIA retrospective and prospective cohorts included significantly more female patients, symptomatic aneurysms, and lesions presenting with SAH than our review did. The retrospective cohort included significantly more cases of large aneurysms but fewer cases in which the lesion was located in the posterior circulation. Compared with our study, the prospective group did not show any differences in these subgroups. The ages of patients who were included could not be compared. The overall rupture rate in the 13 studies we reviewed was significantly higher compared with the rates of both the prospective and retrospective ISUIA cohorts. Our review also showed a higher risk of rupture compared with the review by Rinkel, et al.,[22] whereas the significance was less ($p = 0.02$). Excluding Japanese series from the European study did not change our findings. The European review included more cases of anteriorly located lesions, symptomatic aneurysms, and lesions with SAH.

Discussion

Rupture Rate Associated With UCAs

This systematic review shows that the rupture risk of UCAs observed over time in Japanese institutions is relatively high. Eleven percent of all patients who were included in the articles we reviewed experienced rupture of the aneurysm and the average annual rupture rate was 2.7% (95% CI 2.2–3.3%). An analysis of subgroups by RRs of rupture per 100 patient-years and the OR of cases of rupture versus those of nonrupture showed that significant factors influencing the rupture risk include the size, location, and symptomatic presentation of the aneurysm. Patient sex and age and a history of SAH also affected the risk, but these factors did not reach statistical significance. Papers published in Japanese and papers including fewer cases documented a slightly higher risk of rupture, but no significant difference was recognized. Multiple trials have failed to identify any influence of climate, physical stress, or emotional stress on the rate of SAH.[23] To determine whether there was any influence of climate or environmental stress on the rupture risk of UCAs among Japanese patients, in addition to the geographical location of the institution, we compared studies according to average yearly temperature, the largest difference in the average temperature within a year in the territory of each institution (data extracted from the database of the Japan Meteorological Society, http://www.jma.go.jp/JMA_HP/jma/indexe.html), and the population data (data obtained from the database of the Japan Statistics Bureau, http://www.stat.go.jp/english/index.htm). Japanese people usually seek medical care close to their homes, and thus we assumed that the statistics and climate information of a particular institute reflect the patient's living environment. Our comparison of 12 studies (the 13th was a multicenter study that could not be confined to a single area) revealed no apparent differences in climate or population subgroups. In this review, we could not assess other previously documented risk factors associated with the rupture rate, such as hypertension or smoking. Of note, the prevalence of hypertension or smoking in Japanese adults is not higher than that of the US population.[5,18,21]

By systematically collecting data from case series, we determined the number of patient-years and used that number in our analysis of the data. Although our review showed that there were fewer female patients and fewer patients with SAH or symptomatic aneurysms, overall these characteristics did not significantly differ from those of large cohort studies. The rupture rate documented in our review, however, was significantly higher.[11,12] A comparison with the systemic review by Rinkel, et al.,[22] also showed a difference, but this difference was smaller ($p = 0.02$). Even in the patient group with the least risk (patients with aneurysms < 10 cm), the calculated rupture rate was 1.5% per year in our review (95% CI 1.0–2.15%). In addition, rupture was reported in 11 cases in which the aneurysm was smaller than 7 mm (27.5% of all ruptured cases in the pertinent series).

Race or Bias

To explain the high rupture rate documented in our re-

TABLE 3
Comparison of the current review with international studies

Authors & Year	Total No. of Cases	Patient-Years	No. of Ruptures	% Rupture Rate (95% CI)	p Value*
ISUIA, 1998	1449	12023	32	0.3 (0.2–0.4)	<0.0001
Rinkel, et al., 1998	NA	3907	75	1.9 (1.5–2.4)	0.02
ISUIA, 2003	1692	6544	51	0.8 (0.6–1.0)	<0.0001
present study	922	3801	104	2.7 (2.2–3.3)	

* Fisher exact test.

view, we considered at least two factors. First, UCAs in Japanese patients may have a higher risk of rupture due to a genetic or habitual background. The rate of SAH in the Japanese population is reported to be very high, as high as 96 per 100,000 annually[15]—almost nine times higher than the incidence observed in Rochester, MN (11 per 100,000 annually[10]). Nevertheless, the prevalence of UCAs is not significantly different among the populations studied,[22] which might indicate that UCAs in Japanese patients may rupture more frequently than they do in the Caucasian population. An SAH, however, is not necessarily a consequence of the rupture of a UCA that has already formed. Kataoka, et al.,[14] noted that the pathological analysis of ruptured aneurysms showed evidence of acute formation of the lesion's wall, and some SAHs may be caused by acutely formed aneurysms, which would not be detected as UCAs during routine physical or imaging examinations. Furthermore, the prevalence of SAH may differ according to the study design and how the rate was determined. Nevertheless, the high rupture risk of documented UCAs may explain the high rate of SAH in Japan, and this high risk may relate to the genetic or habitual backgrounds of the Japanese patients.

Secondly, we considered that our review might simply incorporate series that included highly biased cases. Two possible steps could create such a bias in patient selection. Large aneurysms and those located in the posterior circulation are known to be associated with a high risk of rupture.[11] Elderly people are also known to have a higher rate of SAH.[15] These factors elevate surgical risks,[11] which may make surgeons reluctant to perform surgery in patients with these factors. Hence, these patients may be left untreated. Except for the inclusion of more cases with aneurysms of the posterior circulation, however, the characteristics of patients in this review included more patients with lower risk than those found in either the ISUIA cohorts[11,12] or the review written in Europe.[22] Therefore, this bias does not appear to be the main reason for the difference in rupture risk.

As a second bias, we may need to consider intrinsic variations in the design of the studies. Large-scale cohorts, especially in prospective studies, may include all patients who have been encountered, even those seen only in outpatient clinics. On the other hand, retrospective series tend to include patients who were admitted to the hospital for some reason and then evaluated for intervention. These nuances in the selection criteria for patients might produce a bias in the studies. In part of this study (11 series), the number of patients who underwent surgery for UCAs was twice that of patients who were merely observed. Hence, our study does not represent all UCAs that we encountered in our daily practice and should always take into account the selection bias.

Based on these findings, patients with UCAs that were not treated but observed in Japanese institutions may indeed carry a relatively high risk of lesion rupture. These patients, however, might also have high medical risks, surgical risks, or both, and treatment should be chosen according to a detailed risk–benefit analysis for each scenario. Although we cannot currently define the genetic factors influencing rupture, we would like to warn physicians that UCAs might have a different natural course in different races, and the data reported by the ISUIA[11] may not apply to all cases.

To understand the real risks of rupture in all patients with UCAs encountered in daily practice, we should rely on prospective cohort studies conducted in individual countries or on those with an international basis. In Japan, there are two ongoing prospective studies. When these studies are completed, we should be better equipped to determine the natural history of UCAs, whether that history differs among countries or races, and what treatment should be used for patients with UCAs in Japan.

Conclusions

A systemic review of untreated UCAs in Japan showed that the risk of rupture is significantly higher than that reported by international large-scale cohort studies. This difference in the rupture risk might be induced by differences in racial or genetic backgrounds or by differences in study designs or patient backgrounds. Based on these findings, we can state that untreated UCAs in Japan, once excluded from surgical indications, have a significantly high rate of rupture, but this rate may not apply to all patients treated in outpatient clinics.

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See the corresponding article in this issue, pp 601–606.

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Editorial

The risk of rupture of unruptured cerebral aneurysms in the Japanese population: a systematic review of the literature from Japan by Morita, et al.

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Morita and colleagues present a very interesting paper about an important topic that may be particularly pertinent in Japan given that some of the highest incidence rates for subarachnoid hemorrhage (SAH) have been cited in reports from that country. There are obviously many challenges inherent in the approach of collating and combining data from several relatively small retrospective reports, as illustrated by this study and substantially acknowledged by the authors. We faced similar challenges in North America and Europe in an attempt to evaluate small retrospective studies, and our inability to provide uniform, robust results while using this approach led to the development of the International Study of Unruptured Intracranial Aneurysms (ISUIA). It is nevertheless interesting that the results of the current study indicating increased rupture risk for large, posterior circulation, and symptomatic unruptured cerebral aneurysms were very similar to the pattern observed in the ISUIA[1,2] (a multivariate analysis performed in the ISUIA indicated that the increased risk associated with symptomatic unruptured aneurysms was related to the increased size of these lesions). Moreover, the overall rupture rate of 2.7% per year reported in the current study would not differ statistically from the overall rupture rates we reported from early small retrospective series from a single institution.[3,4] It is difficult to evaluate the apparent cases of rupture of small aneurysms in the absence of information about which patients had prior SAH and without sufficient follow-up information to allow calculation of rupture rates. Given the substantial differences in patient populations, study design, and follow-up analyses, it is not statistically possible to compare the results of the current metaanalysis with the results of the ISUIA by using traditional probability values.

Notwithstanding the aforementioned points, the results of the study by Morita, et al., are intriguing and provide food for thought as we anticipate the results of the two ongoing prospective studies in Japan that the authors mention in their paper. A difference in risk factors and the behavior of unruptured intracranial aneurysms in substantially different genetic populations cannot be excluded.

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Response: We appreciate Dr. Wiebers' thoughtful comments about our systematic review. As he has emphasized, reviewing and summarizing small series is difficult because the case material, classification, follow-up methods, and study periods differ among series. Because of these difficulties, we asked the authors of each study included in our review to provide their own data reclassified according to our criteria. Most of the authors kindly fulfilled our request, and we particularly appreciate their cooperation. The strength of our study relies on their efforts, which we could request because our report is based on single-nation studies and we know each other very well. Without such a relationship with each author, we might not have been able to obtain uniformly classified data. Nonetheless, as Dr. Wiebers notes, it was still a difficult task to collect such information because some of the authors' data were already lost—some from a change in recording style occurring during software upgrades and some because of computer breakdowns. Furthermore, some of the older raw data had not been obtained with informed consent from patients and we did not collect raw data. To carry out a multivariate analysis regarding risk factors (such as a comparison of the influence of symptomatic and larger aneurysms), we need patients' raw data. Problems such as publication biases be-

come more serious when assessing management results collected from surgical series.[4] With these problems in mind, we strongly recommend that authors who wish to publish their own series of specific diseases obtain informed consent from each patient for a generalized data analysis. Authors should also keep raw data obtained in each patient with their report in a format that will not be lost. Such efforts can contribute tremendously to future scientific study. Furthermore, the method of classification, measures used to evaluate outcomes or events, and other pertinent information should be uniform. We hope that guidelines developed to direct the management of specific diseases also contain recommendations about methods of follow up and other pertinent issues.[1] The two on-going prospective studies in Japan have been constructed to overcome the innate problems of retrospective data collection. The first study is a prospective on-line collection of data from patients with unruptured cerebral aneurysms treated in the involved institutions (Unruptured Cerebral Aneurysm Study in Japan, UCAS Japan).[2] Each patient chose a treatment plan based on the recommendations of the attending physician, and prospective follow-up and management data are being assessed. No results about rupture risk or management outcome have yet been published. The second study is being conducted by a group of neurosurgeons at national hospitals who agreed to observe all patients harboring unruptured aneurysms with a diameter less than 5 mm (Small Unruptured Aneurysm Verification; SUAVe study). The latest publication from this group[3] shows that, even among these small lesions, four aneurysms ruptured and the calculated rupture risk was 0.8% per year (95 confidence interval 0.2–3%). Eighteen aneurysms enlarged, seven of which were surgically treated. A location on the anterior communicating artery and the occurrence of multiple aneurysms in older women were factors affecting the rupture risk. Because the study is limited to a select group and the follow-up period is short, the confidence interval is wide and longer follow-up periods and further case involvement are required to establish acceptable data. Nonetheless, a close follow-up review with reasonable sensitivity to enlargement of the lesion has proved to be a valid method for managing small aneurysms. We hope such efforts to build valid prospective data obtained via uniform measures from multiple institutions will solve some of the mystery surrounding unruptured aneurysms and provide useful information for their appropriate treatment. This cannot be accomplished using the current retrospective analysis of data. These efforts might also be used to identify the reason for the difference in incidence of SAH between patients in Japan and those in Western countries. Nevertheless, there will still be some patients in whom detailed prospective data analysis may not clarify the optimal management strategy and a randomized controlled trial is required. The aforementioned prospective studies may help us define the group of patients best served by randomized controlled trials.

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3. 未破裂脳動脈瘤—治療適応と到達目標．何がゴールか？

①日本未破裂脳動脈瘤悉皆調査：UCAS Japan の最新知見

日本未破裂脳動脈瘤悉皆調査事務局（東京大学脳神経外科内）
森田　明夫

【目的】UCAS Japan（日本未破裂脳動脈瘤悉皆調査）は未破裂脳動脈瘤の自然歴・治療に関するリスクの検証，データバンクの構築を目的とした前向きコホート研究である．本発表では UCAS Japan 登録例の最新データを報告する．【方法】参加施設において 2001 年 1 月から 2004 年 4 月までの間に発見された治療例・経過観察例すべての未破裂脳動脈瘤の診断時状況，3 カ月・12 カ月・36 カ月における経過観察をインフォームド・コンセント取得後オンライン登録する．【結果】開始 39 カ月（2004 年 6 月）の段階で参加施設は 404 施設，登録症例数は 6,646 例，動脈瘤数は 8,161 個である．男女比は 1：2，中間年齢は 63 歳である．瘤のサイズは 3〜45 mm（中間 5 mm），部位は中大脳動脈（33％），内頸動脈（34％）の順に多く，多発性は 17％であった．瘤発見のきっかけは頭痛やめまいなどの精査で発見されたものが最も多かった．治療は 2,431 例に適応された．【結論】今後さらに症例の追跡を徹底し，未破裂脳動脈瘤の治療方針決定に資するデータ構築を目指す．

Stroke

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Impaired Progression of Cerebral Aneurysms in Interleukin- 1β-deficient Mice

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Background and Purpose- Subarachnoid hemorrhage caused by cerebral aneurysm rupture remains a life-threatening emergency despite advances in treatment. However, the mechanisms underlying aneurysm initiation, progression and rupture remain unclear. We have developed a method to induce experimental cerebral aneurysms in rats, monkeys, and mice. Interleukin-1β is a key inflammatory mediator and it is thought to be a promising target for the treatment of inflammatory diseases. In the present study, we examined the role of IL-1β in cerebral aneurysm development.

Methods- Cerebral aneurysms were experimentally induced in five week-old male C57BL/6 mice, IL-1β gene deficient mice (IL-1β-/-) and age-matched control B10 mice (wild-type). Their cerebral arteries were dissected and examined histologically and immunohistochemically.

Results - IL-1β was expressed in vascular media in mice at early stage of anerysmal models' cerebral arteries. No differences were seen in the rate of aneurysm development between IL-1β-/- and wild-type mice, but the percentage of advanced aneurysm change was significantly larger in wild-type animals. Furthermore, in IL-1β-/- mice, increased caspase-1 expression was seen compared to wild-type animals. Additionally, the number of apoptotic cells assessed by ssDNA immunoreactivity and TUNEL was significantly reduced in IL-1β deficient mice compared to wild-type animals.

Conclusions - IL-1β is important for the progression of cerebral aneurysms in a mouse model. Disruption of the IL-1β gene results in the reduced incidence of mature experimental cerebral aneurysms.

Cerebral aneurysm rupture with subsequent subarachnoid hemorrhage remains a life-threatening medical emergency despite recent diagnostic and therapeutic advances. The mechanisms of aneurysm initiation, progression, and rupture remain unclear, however (1). Most studies examining the pathophysiology of cerebral aneurysm have relied upon specimens obtained during autopsy or surgery. These samples are not suitable for examining the molecular factors leading to aneurysm initiation and progression.

We developed a novel experimental model of cerebral aneurysms in rats, monkeys, and mice (2-6), quite resemble to human cerebral aneurysms in their anatomic location and histological structure (3,5), and it allowed us to examine the early stages of cerebral aneurysm development in greater detail.

We previously found extensive apoptotic cell death of smooth muscle cells in the aneurysmal walls using this model system (7). Additionally, using inducible nitric oxide synthase (iNOS) inhibitor and iNOS deficient mice, we demonstrated that iNOS is important in cerebral aneurysm progression (8,9). Interleukin-1 (IL-1) is a proimflammatory cytokine highly produced by mononuclear phagocytes, and to a lesser extent by other cell types, in response to injury and infection (10,11). IL-1β is produced as an inactive precursor protein that is proteolytically cleaved by an endoproteinase originally termed IL-1β-converting enzyme (ICE), which was subsequently found to be caspase-1 (12). IL-1 functions in a broad array of normal and pathological inflammatory, hematopoietic, and immunologic situations. IL-1 is thought to be a key inflammatory mediator, and it is a potential target for therapy in inflammatory diseases. (10,11). Additionally, IL-1β is a potent iNOS activator (13,14).

In the present study, we examined the distribution of IL-1 in cerebral aneurysmal development using an experimental model in mice.

Materials and Methods

Induction of Experimental Cerebral Aneurysms

Seven week-old male C57BL/6 mice were purchased from Shimizu-jikken-doubutsu (Kyoto Japan), and 5-6 week-old IL-1β gene deficient mice (IL-1β-/-) (15) and age-matched control B10 mice (wild-type) were purchased from Taconic (Petersburgh, NY). Cerebral aneurysms were induced using the procedure reported by Morimoto et al (6). The left common carotid artery and posterior branch of the left renal artery were ligated with 10-0 nylon under general anesthesia with 1% to 2% halothane. One week later, another side was operated. The mice were fed 8% sodium chloride and 0.12% BAPN containing food. Sodium chloride was used to enhance the degree of hypertension. BAPN is an inhibitor of lysyl oxidase, the enzyme that catalyzes the cross-linking of collagen and elastin, and was used to increase vessel fragility. Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

RNA isolation and reverse transcription

2 weeks and 3 months after the operation of the C57BL/6 mice and 5 months after the operation of the IL-1β gene deficient mice (IL-1β-/-) and age-matched control B10 mice, total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit from Qiagen (Hilden, Germany). Extraction was performed according to the manufacturer's directions. 10ng of total RNA were converted to cDNA using Sensiscript reverse transcriptase (Qiagen). The conditions for the cDNA synthesis were: 60 min at 37°C followed by heating at 93°C for 5 min. 2μl of the samples were used in PCR reaction using HotStarTaq polymerase (Qiagen). PCR was started at 94°C for 15 min, followed by 45 cycles (94 °C for 1min , 53 °C for 1 min, and 72 °C for 1 min), ended with a 10-min incubation at 72 °C for Caspase-1. In case of GAPDH and β-actin, cycles were 40, and IL-1β were 45cycles (94 °C for 20sec, 55 °C for 45 sec, and 72 °C for 40sec). Primers were as follows: caspase-1, forward

5'-GAGAGGAGAGTGCTGATTCAGG-3' and reverse 5'-CAAGACGTGTACGAGTGGTTGT-3', product size 400 bp; GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3', product size 452 bp; IL-1β, forward 5'-GGTGTGTGACGTTCCATTAGA-3' and reverse 5'-CATGGAGAATATCACTTGTTGGTTGA-3', product size 143 bp; β-actin, forward 5'-GTATGCCTCGGTGGTACCA-3' and reverse 5'-CTTCTGCATCCTGTCAGCAA-3', product size 499 bp. GAPDH and β-actin were used as a control and the PCR products were separated by electrophoresis in 2% agarose gels.

Tissue Preparation

Five months after aneurysm induction, animals were deeply anesthetized and perfused transcardially with physiological saline followed by 4% paraformaldehyde at a speed of approximately 0.7 mL/h using a peristaltic pump (RP-2000, EYELA, Tokyo). The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation and another aneurysm susceptible portion were stripped and embedded in OCT compound (Tissue-Tek, Sakura fine technical co., Tokyo, Japan). 4 μm semithin sections were cut and mounted on silane-coated slides. We could make about 10 slides per one lesion.

Definitions

Aneurysm, as defined here, refers to an outward bulging of the arterial wall detected by light microscopy (9). *Early aneurysmal change* refers to a lesion with discontinuity of the internal elastic lamina visualized by orcein stain without apparent outward bulging of the vascular wall (Fig1 g and h). *Advanced cerebral aneurysm* refers to an obvious outward bulging of the arterial wall with fragmentation and disappearance of the internal elastic lamina (Fig1 i). Three independent researchers assessed the histopathological changes and selected the section at the maximum diameter of each aneurysm in a blinded manner.

Orcein staining and size measurement

Specimens were washed and incubated in orcein solution (orcein 0.1g, 70% Ethanol 100 ml, 35% HCl 2 ml) for 24 h. After dehydration, delipidation and enclosure, all samples were classified into 3 groups; *No Change* group (NC), *Early aneurysmal change* group (EAC) and *Advanced cerebral aneurysm* group (AA).

Immunohistochemistry

Slides were then washed 3 times with PBS containing 0.1% Tween 20 (PBS-T) and incubated with secondary antibody (anti goat Alexa-Fluor 488 antibody and anti mouse Alexa-Fluor 546 antibody; Molecular Probes, Eugene, OR) for 1 h at room temperature. Mouse staining was performed using rabbit polyclonal anti single-stranded DNA antibody (1:500; DAKO, Glostrup, Denmark) or rabbit polyclonal anti Caspase-1 antibody (1:200; Santa-Cruz, Santa Cruz, CA) and Cy3-conjugated mouse monoclonal anti-α-smooth muscle actin antibody (Sigma, St. Louis, MO) as primary antibodies. The sections were incubated with primary antibody solutions overnight at 4°C. The slides were then washed 3 times with PBS-T and subsequently incubated with biotinylated anti-rabbit IgG, 1:250 (Vector Laboratories, Burlingame, CA) for 1 h at room temperature (RT). After three more washes with PBS-T, Alexa Fluor Fluoronanogold streptavidin 488 (Molecular Probes) was applied for 30 min at RT. Slides were washed 3 more times with PBS-T and covered with PERMAFLUOR (Immunotech, Marseille, France) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1, Olympus, Tokyo, Japan). The anti-IL-1β antibody does not cross-react with IL-1α isoforms.

TUNEL

Cell death was detected in situ by enzymic labelling of DNA strand breaks using an In Situ Cell

Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. And co-stained with Cy3-conjugated mouse monoclonal anti-α-smooth muscle actin antibody (Sigma, St. Louis, MO) as previously discribed.

Cell Counting

The observer captured the image under microscope from total sections including aneurysm from one animal (three to five). Image-Pro plus (Media Cybernetics; San Diego, CA) was used for photo imaging and cell counting. Mean number of positive cells per section was recorded.

Statistical Analysis

The values were expressed as means ± SD. Statistical analysis was performed using χ^2 for independence test for aneurysm formation rate, Mann-Whitney's U test for advanced aneurysm change and Welch's t-test for others. Differences were considered statistically significant at $P<0.05$.

Results

IL-1β expression in aneurysmal walls of mouse cerebral aneurysm

We examined IL-1β expression in the aneurysmal vessel walls by immunohistochemical method. Double staining for α-actin (Fig. 1 red, b and c) and interleukin-1β indicated that IL-1β was expressed in medial smooth muscle cells (green b and d). Next we analyzed the expression of IL-1β at 2 weeks and 3 months after the surgery. In our model, the typical EAC and AA histology was observed at 2 weeks and 3 months, respectively. RT-PCR (Fig 1 e and f) revealed that 2 weeks groups (n=3) had significantly higher expression of IL-1β mRNA than controls (n=4, P=0.003) and 3 months groups (n=5, P=0.004).

Characteristics of experimental cerebral aneurysms in IL-1β deficient mice

Five months after surgery, we analyzed cerebral aneurysmal changes in IL-1β deficient mice (n=11) and wild-type controls (n=13). Examining the wild-type mice, we identified four animals with advanced aneurysmal changes (AA Fig1 i), four animals with early aneurysmal changes (EAC Fig1 g and h), and five mice with no changes (NC). In contrast, when the IL-1β-/- animals were examined, no AA lesions were found, six mice had EAC, and five animals NC animals. As shown in Figure 2-a, the rate of the aneurysmal change is not different between the wild-type and IL-1β deficient mice (p=0.527). However, the rate of advanced aneurysm per total aneurysmal change (AA + EAC) was significantly larger in wild-type controls than in IL-1β deficient mice (p=0.048). Mean blood pressure of the two groups did not show significant difference before the surgery (wild-type 97.03 ±4.27 to IL-1β-/- 92.16±6.65; P=0.20) and after the surgery (wild-type 129.22 ±8.76 to IL-1β-/- 130.11±10.16; P=0.89).

Expression of caspase-1 and single-stranded DNA in IL-1β deficient mice

Given the differences in aneurysm progression in wild-type and IL-1β-/- animals, we wished to examine other pathways downstream of IL-1β. We analyzed the expression of caspase-1, which activates pro-IL-1β, single-stranded DNA (ssDNA) and TUNEL, which are as markers of apoptosis. We observed increased caspase-1 immunoreactivity in IL-1β deficient mice compared to wild-type mice and were most pronounced in the media (Fig. 3). We next examined the apoptosis in the aneurismal walls. The cells expressing ssDNA and TUNEL were quantified with Image-Pro plus for comparison of IL-1β-/- and wild-type animals. ssDNA immunopositive and TUNEL-positive cells were primarily found in the media, and reduced number of positive cells was seen in IL-1β-/- mice compared to wild-type animals (Fig. 4). The total number of ssDNA positive cell was 27±9.24 (n=5) and TUNEL-positive cells was 30.2±10.6 in the wild-type group and 10±4.32 (n=4), and 3.6±3.78

(n=5) in the IL-1β-/- group. These differences were significant (p=0.027, p=0.0033, respectively). Furthermore, significantly more smooth muscle cells contained ssDNA and TUNEL in the wild-type group (14.7±7.70 cells and 14.6±3.65 cells, respectively) compared to the IL-1β knock-out mice (2.75±2.63, p=0.049 and 0.6±0.55, p= 0.0011, respectively). No differences were seen between the number of ssDNA positive endothelial cells (EC) between wild-type (4.7±3.27) and knock-out mice (2.75±2.63, p = 0.079).

Discussion

Using an experimental model of cerebral aneurysms that closely mimics the histopathology seen in human lesions, we have clearly shown that IL-1β is induced in the aneurysmal walls during the early stages of aneurysm development. Moreover, animals deficient in IL-1β exhibit delayed aneurysm progression compared to wild-type mice. An inability to produce IL-1β also led to increased caspase-1 expression but decreased ssDNA immunoreactivity. Taken together, these data indicate that IL-1β expression in smooth muscle cell promotes SMC apoptosis and this may enhance aneurysm formation.

Since Hashimoto et al. originally described the experimental induction of cerebral aneurysms in rats (2), we have analyzed the mechanisms of cerebral aneurysm formation using rats and monkeys (2-6). We confirmed that SMCs in the arterial walls of aneurysms die via apoptosis (7,16). In addition, we reported that iNOS was markedly induced in human and rat cerebral aneurysms, and aminoguanidine, a relatively selective iNOS inhibitor, suppressed the incidence of experimental cerebral aneurysms in rats (8). Recently, Morimoto et al. (6) developed a cerebral aneurysm model in mice, we showed that both cerebral aneurysm size and the number of apoptotic vascular smooth muscle cells in iNOS deficient mice were significantly reduced compared to wild-type littermates (9). Activation of iNOS depends on cellular exposure to immunologic or inflammatory stimuli such as bacterial endotoxins,