2. Ross手術：自己肺動脈弁を用いた大動脈弁置換術

響庭 了

[慶應義塾大学医学部外科（心臓血管）]

はじめに

Ross手術はautograft（自己肺動脈弁）を用いた大動脈弁置換手術であり，1967年Donald Rossが報告した方法である[1]．大動脈弁置換手術のうちで唯一，生きている弁を用いる手術である．したがって，人工弁の植込み後に生じうる，弁stuck，血栓塞栓症，出血，溶血，感染などの重篤な合併症がなく，またその弁の血行動態上の性能はあらゆる人工弁を上回り，また小児では弁構造の成長が見込めるという数々の利点を持っている．一方で，手術手技が複雑になることに伴う術後早期リスク，弁機能の耐久性，autograft基部の遠隔期の拡張，肺動脈弁位に植え込んだ代用弁の耐久性（ただし閉鎖不全は長期に耐えうる）といった数々の懸念がある．したがってその適応を決定する際にはこれらの功罪を考慮に入れて対処すべきである．現在，Ross手術の適応に関しては，積極的に小児から50歳以上までの幅広い年齢，背景疾患を対象とするElkins[2]や10歳代の若者を含め可及的に行わない方針としているとするJonas[3]まで専門家間で意見が大きく分かれている．しかし，小児の大動脈弁置換においては本術式が第一の適応となる．以前はKonno手術などの人工弁を用いた弁輪拡大術が多く行われたが，現在ではRoss手術が行われることが多くなった．新生児乳児においても適応となる場合がある．ワルファリン服用が不要なため，妊娠可能年齢の女性や，運動家などの活動性の高い患者がよい適応である．また感染性心内膜炎による大動脈弁疾患にも適応が存在する．

1. 手術手技

胸骨正中切開にて心臓に到達し，遠位上行大動脈送血，上下大静脈脱血によって完全体外循環を確立する．

まず，心拍動下で，autograftの採取を開始する．主肺動脈を最も遠位部すなわち左右分枝肺動脈との接合部で横切断することにより主肺動脈壁のすべてをautograft側につけて採取する．なぜなら主肺動脈の長さは個体差がありこれが短いケースでは前側の交連を手術などで構造物が癒着している場合などは左右分枝肺動脈との接合部に最初に目印をつけておくとよい．

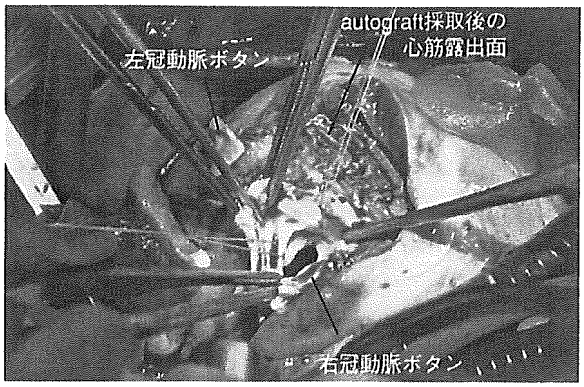
著者らは，この時点で心停止を導入する．上行大動脈を遮断し，心筋保護液を順行性に注入する．右室流出路の横切開の時には，切断した主肺動脈から肺動脈弁輪を確認し，直角鉗子を入れて弁輪の最下部から正確に2～3mmのレベルで始める．心室中隔の切開では左冠動脈の損傷しないように十分に留意する．左前下行枝はautograftの後側を走行しているが，再手術による癒着などで左前下行枝が視認しづらい時には，大動脈をST接合部よりやや遠位で横切断し，左冠動脈口からプローベを左前下行枝に挿入してこれを心外膜側より触知する．第一中隔枝は三尖弁の内側乳頭筋基部を通る横隔膜からの垂線に一致して位置している[4]．心室中隔は右室側から見て第1層と第2層の間を切離していく．筋束走行が変化する深さが筋層間であり，この切開線のうち大動脈と肺動

脈との接合部でのみ線維弁輪骨格を切離する．

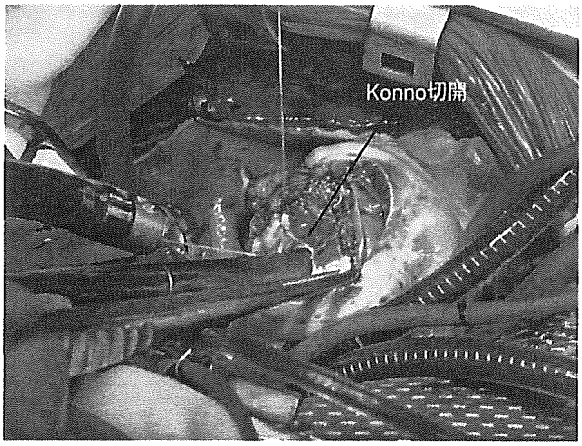
大動脈弁の狭小弁輪のためにRoss-Konno手術を行う時には右室自由壁をU字型に切開しこの部分がautograft側に付着するようにする．採取したautograftは生理食塩水を満たして，弁の接合を確認し，弁輪のサイズをHegar dilatorで測定した後，心嚢内でice slushを用いて保存しておく．

autograftの植込み術式は，基部置換法，subcoronary法，シリンダー内挿法などがあるが，2005年現在，最も普及していると思われる基部置換法について述べる．

左右の冠動脈ボタンを大きめのカフをつけて作成しておく．続いて大動脈弁尖を切除する（図1）．そして大動脈弁輪のサイズをHegar dilatorで測定する．前に測定したautograftのサイズと±2 mm程度の差であれば弁輪径の調整手技は必要ない．弁輪径を拡大する時には，左冠尖-右冠尖間の交連部で心室中隔を切開するKonno切開（図2）を加える（Ross-Konno手術）．また弁輪径を縮小する時には，弁輪外周に（目標弁輪外径×π）の長さのdacron felt帯を縫着する（annulus reduction）．また，成人においては弁輪径の調整が不要な場合であっても，遠隔期の弁輪拡張を予防する意味で弁輪外周の長さのdacron felt帯を縫着する（annulus fixation）．まず第1列の針糸のうち，autograft弁輪最下部（nadir）3点を対応する大動脈弁輪にかける．この時，大動脈弁は必ずしも120°対称となっていないことがあるので，大動脈弁輪にかける位置を調整して，autograftが歪まないように留意する．autograftはRoss手術の場合は120°反時計回りに回転させてautograft前尖（右室流出路との連続部分）を無冠尖部分になるようにし，Ross-Konno手術の場合は回転させずにautograft前尖と右室流出路の延長部分がKonno切開にはまるように位置させる（図3）．大動脈弁輪に対してscallopではなくできるだけ円形となるように交連部では左室内膜面にかける．dacron felt帯を縫着する時にはこの針糸を巻き込むようにかけていく．この時，



［図1］autograft採取，大動脈弁切除，と左右冠動脈ボタンの作成



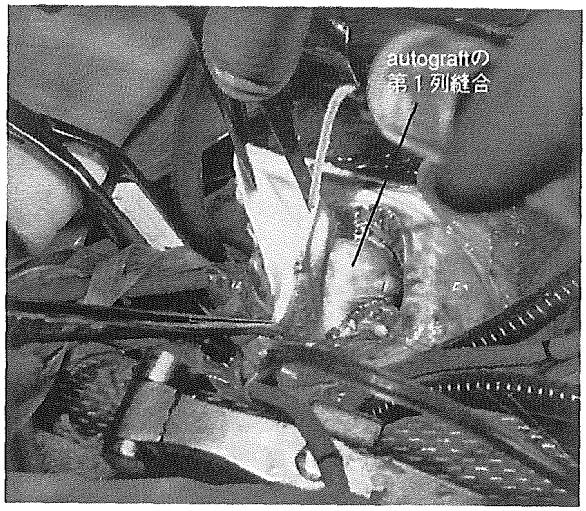
［図2］Konno切開による弁輪拡大

autograftをいったん左室内にinvertすると視野が改善することがある．次に左冠動脈を再建する．autograftのValsalva洞内に冠動脈ボタンより小さい孔をあけて，冠動脈ボタンを縫合する．続いて上行大動脈-autograft吻合を行う．上行大動脈が拡大している時には人工血管置換や大動脈壁縫縮を追加するが，autograft側は切り込みを入れないようにする．最後に右冠動脈ボタンの吻合する正確な位置を決定して左冠動脈と同様の方法で再建する．

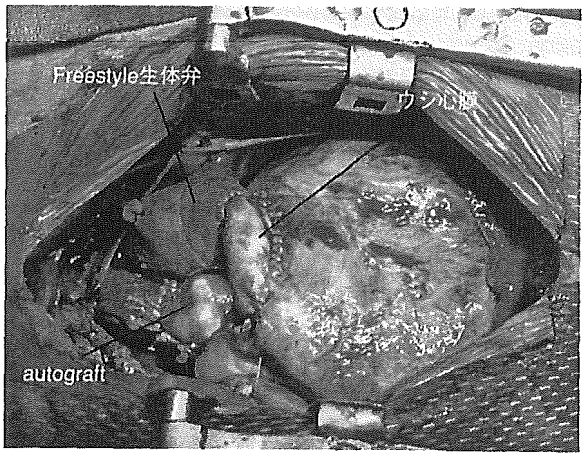
続いて右室流出路の再建に移る．最適の材料はpulmonary valved homograftであるが，本邦においては入手が困難であり，他の材料を用いざるを得ないことが多い．著者らは主としてMedtronic Freestyle異種生体弁を用いている．

◎autograft 採取の際，左冠動脈の損傷を避ける．
◎基部置換法では第1列の針糸は scallop 型ではなく円形に配列するようにかける．
◎homograft が入手できない場合でも右室流出路再建には異種生体弁などで対応が可能である．

まず心停止の間に autograft を採取した後の心室中隔の断端は心内膜の欠如した心筋組織露出面であり，左冠動脈または第一中隔枝からの小枝からの出血を予防するために心筋保護液を注入して，電気メスなどで十分に止血しておく．homograft が入手できる場合には右室心内膜と直接縫合しても，内膜組織の裂開が生じることはない．Medtronic Freestyle 異種生体弁を含めて，他の材料では柔軟性に問題があるので，まず心筋組織露出面全体を自己心膜で覆って閉鎖しておき，この自己心膜に生体弁を縫合する．右室の中隔側との吻合が終了して大動脈の遮断を解除する．心拍動の再開を確認した後，右室前壁の縫合を行う．Ross-Konno 手術を行った時には，ここにさらに異種心膜などの補填を必要とすることが多い．主肺動脈と吻合の端々吻合を行って，心内操作を完了する（図4）．



[図3] autograft の基部置換法による植込み操作（第1列終了）



[図4] Ross-Konno 手術の完成図

おわりに

最近の動向として，大動脈弁閉鎖不全に対しての適応を慎重に考える意見が増えていることと，autograft の植込み方法も遠隔期の autograft 拡張を防止する観点から，Ross 原法の subcoronary 法やシリンダー内挿法が復活していることがあげられる．

文献

1) Ross, DN : Replacement of aortic and mitral valves with a pulmonary autograft. Lancet 2 : 956, 1967
2) Elkins, RC : The Ross operation : a 12-year experience. Ann Thorac Surg 68 (3 suppl) : S14-18, 1999
3) Jonas, RA : The Ross procedure is not the procedure of choice for the teenager requiring aortic valve replacement. Semin Thorac Cardiovasc Surg Pediatr Card Surg Ann 8 : 176-180, 2005
4) Hosseinpour, AR, Anderson, RH, Ho, SY : The anatomy of the septal perforating arteries in normal and congenitally malformed hearts. J Thorac Cardiovasc Surg 121 : 1046-1052, 2001

J Infect Chemother (2005) 11:278–287
DOI 10.1007/s10156-005-0413-z

ORIGINAL ARTICLE

Naoki Aikawa · Shigeru Kohno · Kazutoshi Shibuya
Yoshio Takesue · Shigefumi Maesaki · Minoru Yoshida
Issei Tokimatsu

Current status of diagnosis and treatment of invasive fungal infections in Japan: the influence of the new Japanese guidelines

Received: August 29, 2005 / Accepted: October 7, 2005

Abstract One year after the release of the Japanese "Guidelines for the Diagnosis and Treatment of Deep-Seated Mycosis" we conducted a survey of clinicians to determine the extent to which the new guidelines had penetrated clinical practice, and compared these results with those of a previous survey. The responses to the current survey regarding the diagnosis and treatment had changed from those of the previous survey to reflect the new recommendations, showing that the guidelines have had an effect on clinical practice. However, the current survey highlights a need to provide more practical information in the guidelines for use in the clinical settings. In particular, physicians expect the guidelines to proactively provide more information about the features of both current and new drugs. In addition, an effective drug against the genus *Aspergillus* is eagerly awaited. However, because it is difficult to differentiate among filamentous fungi, there is a need for a drug with broad-spectrum coverage against filamentous fungi. Investigation of combination therapy consequently becomes necessary. Definitive diagnoses of invasive fungal infection are too scarce at the national level. The cooperation of clinicians for organizing more definitive diagnoses would be appreciated when the guidelines are revised.

Key words Invasive fungal infection · Guidelines · Diagnosis · Treatment

N. Aikawa (✉)
Keio University Hospital, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
Tel. +81-3-3353-1368; Fax +81-3-3226-9877
e-mail: aikawan@sc.itc.keio.ac.jp

S. Kohno
Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

K. Shibuya
Department of Surgical Pathology, Toho University Faculty of Medicine, Tokyo, Japan

Y. Takesue
First Department of Surgery, Hiroshima University Faculty of Medicine, Hiroshima, Japan

S. Maesaki
Department of Infectious Diseases and Infection Control, Saitama Medical School, Saitama, Japan

M. Yoshida
Fourth Department of Internal Medicine, Teikyo University School of Medicine, Kanagawa, Japan

I. Tokimatsu
Department of Infectious Diseases, Oita University Faculty of Medicine, Oita, Japan

Introduction

There has been a recent increase in the worldwide prevalence of invasive fungal infections.[1,2] In the past, systemic invasive fungal infections were largely associated with complications in hematology, oncology, and organ transplantation. However, in recent years it has become a concern in surgical patients and immunocompromised hosts, and has notably increased in the emergency and intensive care settings. The diagnosis and treatment of invasive fungal infections, which has always been difficult, has become even more of a challenge. In the past, the causative organisms were mainly *Candida albicans*, but in recent years other *Candida* species, *Aspergillus* species, and others have also emerged as causes of invasive fungal infections.[3,4]

Guidelines for management of fungal infections have been established by both the Infectious Diseases Society of America[5] and the European Organization for Research on Treatment of Cancer.[6] However, these are not directly applicable to the current situation in Japan. They are difficult to apply to patients without a definitive diagnosis. Furthermore, some of the recommended antifungal agents are not available in Japan. The guidelines are also limited in that they focus on fungal infections mainly in hematology and organ transplantation. Consequently, there is a need for guidelines that take into account the clinical situation in Japan.

In February 2003, the Deep-Seated Mycosis Guidelines Editorial Committee of the Mycoses Forum released the

"Guidelines for the Diagnosis and Treatment of Deep-Seated Mycosis."[7] Compared with the American or European guidelines, these are based on more pragmatic considerations. First, diagnostic and treatment methods are directed toward the actual context of each diagnostic and treatment setting, including hematology, pulmonary medicine, emergency and intensive care, and surgery. Diagnostic categories are subdivided into three stages: a possible fungal infection, a clinically documented fungal infection, and a proven fungal infection. These are then categorized for therapy as empiric therapy and targeted therapy, respectively. This management algorithm was organized in a flowchart, which, along with other information, was presented for the use of specialists as well as general clinicians.

One year after the release of the guidelines, their acceptance and application were evaluated by a survey taken during the 5th Annual Meeting of the Mycoses Forum, held in January 2004. During the interactive session of the Forum, clinicians who frequently treat patients with fungal infections were asked to complete a questionnaire asking about the guidelines in general and the application of the guidelines. The survey also included questions about details of diagnostic methods used by the clinicians, timing of initiation of therapy, duration of therapy, and other details of treatment. The results of this survey were intended to shed light on the current state of diagnosis and treatment of invasive fungal infections, and to identify the influence of the guidelines on the practice in treating invasive fungal infections.

Material and methods

On 31 January 2004, at the Interactive Session of the 5th Annual Meeting of the Mycoses Forum (Group A), the 28-item questionnaire was presented to the participants of the meeting. There were 72 participants in the session. The participants' answers were immediately collected by auto-analyzers and the total number calculated as a percentage of every answer selector and instantly displayed. For comparison, also displayed were results of the questionnaire survey conducted at the Consensus Meeting (historical control) held during the 72nd Annual Meeting of the Japanese Association for Infectious Diseases in April 1998. For this survey, 47 participants answered the questionnaire.

This report also presents, as referent values, the results of the questionnaire survey to 58 participants conducted during the Oita Prefecture Deep-Seated Mycosis Workshop (Group B) held in Oita Prefecture on March 16, 2004. The questions and answer alternatives were almost the same in all three surveys.

Results

Each question in the surveys is followed by the answers as tabulated.

1. Participants' background

Q.1 What is your specialization?

	Group A	Group B	Group A+B	Historical Control
Hematology	7%	14%	10%	
Pulmonary medicine	28%	26%	27%	
General medicine	7%	17%	12%	No data available
Infectious disease, HIV	14%	2%	8%	
Surgery	1%	20%	10%	
Emergency and/or intensive care	6%	12%	8%	
Organ transplantation	3%	0%	2%	
Other specialties (pediatrics, obstetrics and gynecology, ophthalmology, etc)	2%	11%	6%	
Pathology, laboratory medicine, and bacteriology	30%	–	17%	

2. Frequency of *Candida* species

Q.2 What are the main causative species of deep candidiasis?

	Group A	Group B	Group A+B	Historical Control
Mostly *C. albicans*	73%	53%	64%	64%
C. albicans and *C. glabrata*	13%	25%	18%	14%
C. albicans and *C. parapsilosis*	7%	3%	6%	5%
Various kinds of *Candida* species	6%	3%	5%	12%
Other	1%	0%	1%	0%
Unknown	0%	16%	7%	5%

Q.3 What is the percentage of deep candidiasis among all the infectious diseases that you faced?

	Group A	Group B	Group A+B	Historical Control
Almost nonexistent	39%	49%	43%	
Accounts for around 10% of the total	41%	42%	41%	
Accounts for around 25% of the total	7%	7%	7%	No data available
Accounts for around 50% of the total	6%	0%	3%	
Greater than 50% of total	0%	0%	0%	
Unknown	7%	2%	5%	

3. Diagnostic criteria of deep candidiasis

Q.4 In cases of continuing fever refractory to broad-spectrum antibacterials, after how many days will antifungal drugs be started?

	Group A	Group B	Group A+B	Historical Control
7 days	30%	35%	33%	12%
5 days	31%	28%	29%	8%
4 days	9%	5%	7%	24%
3 days	18%	15%	17%	40%
Other	12%	17%	14%	16%

Q.5 Which method is most important for early diagnosis of deep candidiasis? (up to 2 answers)

	(percentage of the 1st) Group A	Group B	Group A+B	Historical Control
Blood culture (and/or catheter tip culture)	34%	36%	35%	10%
Surveillance cultures	10%	5%	9%	9%
Serological tests	42%	53%	45%	72%
Gene diagnosis (PCR)	13%	4%	10%	9%
Eundoscopy	2%	0%	1%	–
Other	0%	2%	1%	0%

Q.6 In case of positive serological tests for *Candida* and in relation to surveillance culture results, when will empiric antifungal treatment be started?

	Group A	Group B	Group A+B	Historical Control
Positive surveillance cultures – 3 sites or more	15%	0%	8%	
Positive surveillance cultures – 2 sites or more	23%	9%	16%	
Positive surveillance cultures – 1 sites or more	28%	19%	24%	No data available
In case of positive serological test, the therapy is initiated regardless of the number of positive sites at surveillance cultures	34%	72%	52%	

Q.7 In case of negative serological tests for *Candida* and in relation to surveillance culture results, when will empiric antifungal treatment be started?

	Group A	Group B	Group A+B	Historical Control
Positive surveillance cultures – 3 sites or more	27%	11%	20%	
Positive surveillance cultures – 2 sites or more	36%	33%	35%	
Positive surveillance cultures – 1 sites or more	18%	42%	28%	No data available
In case of negative serological test, the therapy is not initiated regardless of the number of positive sites at surveillance cultures	19%	15%	17%	

4. Treatment with antifungals in patients with a positive blood culture to Candida

Q.8 In patients with positive blood culture for *C. albicans*, what drug will be selected as first choice?

	Group A	Group B	Group A+B	Historical Control
Injectable amphotericin B	3%	0%	2%	5%
Oral fluconazole	7%	3%	5%	0%
Injectable fluconazole (*including injectable fosfluconazole)	81%*	95%*	87%*	93%
Oral itraconazole	1%	0%	1%	0%
Injectable miconazole	0%	0%	0%	2%
Oral flucytosine	0%	0%	0%	0%
Injectable micafungin	8%	2%	5%	**

**This arm was not available in the historical control questionnaire.

Q.9 When injectable fluconazole (including injectable fosfluconazole) is selected as first choice for patients with blood culture positive for *C. albicans*, what is the daily maintenance dosage used for adults?

	Group A	Group B	Group A+B	Historical Control
50 mg/day	0%	0%	0%	0%
100 mg/day	3%	5%	4%	0%
200 mg/day	40%	38%	39%	34%
400 mg/day	53%	57%	55%	59%
More than the above indicated dosages	3%	0%	2%	2%
Do not select fluconazole (fosfluconazole)	1%	0%	1%	5%

Q.10 When injectable micafungin is selected as first choice for patients with blood culture positive for *C. albicans*, what is the daily maintenance dosage used for adults?

	Group A	Group B	Group A+B	Historical Control
50 mg/day	1%	7%	4%	
75 mg/day	3%	4%	3%	
100 mg/day	10%	7%	9%	
150 mg/day	51%	23%	38%	
300 mg/day	18%	29%	23%	No data available
More than the above indicated dosages	0%	0%	0%	
It is not selected as first choice	17%	30%	23%	

Q.11 In patients with positive blood culture for *C. parapsilosis*, what drug is selected as first choice?

	Group A	Group B	Group A+B	Historical Control
Injectable amphotericin B	14%	4%	9%	5%
Oral fluconazole	0%	0%	0%	0%
Injectable fluconazole (*including injectable fosfluconazole)	48%*	92%*	68%*	93%
Oral itraconazole	6%	0%	3%	0%
Injectable miconazole	0%	0%	0%	2%
Oral flucytosine	0%	0%	0%	0%
Injectable micafungin	31%	4%	19%	**

**This arm was not available in the historical control questionnaire.

Q.12 In patients with positive blood culture for *C. glabrata*, what drug is selected as first choice?

	Group A	Group B	Group A+B	Historical Control
Injectable amphotericin B	25%	9%	18%	40%
Oral fluconazole	0%	0%	0%	0%
Injectable fluconazole (*including injectable fosfluconazole)	13%*	65%*	38%*	58%
Oral itraconazole	3%	0%	2%	0%
Injectable miconazole	1%	0%	1%	2%
Oral flucytosine	0%	0%	0%	0%
Injectable micafungin	58%	26%	42%	**

**This arm was not available in the historical control questionnaire.

Q.13 When injectable fluconazole (*including injectable fosfluconazole) is selected as first choice for patients with positive blood culture for *C. glabrata*, what is the daily maintenance dosage used for adults?

	Group A*	Group B*	Group A*+B*	Historical Control
50 mg/day	0%	0%	0%	0%
100 mg/day	0%	2%	1%	0%
200 mg/day	3%	18%	11%	16%
400 mg/day	41%	73%	56%	55%
More than the above indicated dosages	31%	0%	16%	2%
It is not selected as first choice	25%	7%	16%	27%

Q.14 When injectable micafungin is selected as first choice for patients with blood culture positive for *C. glabrata*, what is the daily maintenance dosage used for adults?

	Group A	Group B	Group A+B	Historical Control
50 mg/day	0%	4%	2%	
75 mg/day	0%	4%	2%	
100 mg/day	6%	11%	8%	
150 mg/day	72%	36%	56%	
300 mg/day	16%	0%	9%	No data available

Greater than the above indicated dosages	0%	0%	0%
It is not selected as first choice	6%	46%	24%

5. Indications on completion of antifungal therapy against candidemia and disseminated candidiasis

Q.15 Which is the first criterion taken into consideration when completing antifungal therapy against candidemia and disseminated candidiasis in patients with underlying disease?

	Group A	Group B	Group A+B	Historical Control
Culture results	6%	6%	6%	0%
Serological test	25%	29%	27%	7%
Fever and other clinical symptoms	48%	56%	51%	85%
Image findings by CT, echo, etc.	14%	2%	8%	–
Amelioration of the underlying disease (restoration of the number of neutrophils, etc.)	5%	8%	7%	4%
Other	2%	0%	1%	4%

Q.16 On what day do you assess the efficacy of antifungal therapy against candidiasis?

	Group A	Group B	Group A+B	Historical Control
Third day	14%	9%	11%	
Fifth day	27%	22%	25%	
Seventh day	35%	49%	43%	
Tenth day	5%	4%	4%	
Fourteenth day	11%	15%	13%	No data available
A longer time	0%	0%	0%	
Other (it varies according to the degree of severity, other parameters included)	8%	2%	5%	

Q.17 In candidiasis patients with underlying diseases, for how long is the antifungal therapy continued after the disappearance of the clinical signs and symptoms?

	Group A	Group B	Group A+B	Historical Control
After the disappearance, the administration is promptly stopped and only follow-up is conducted	2%	4%	3%	0%
After the disappearance, the administration is continued for 2–3 days	22%	42%	32%	33%
After the disappearance, the administration is continued for around a week	49%	40%	44%	56%
After the disappearance, the administration is continued for 10–14 days	24%	9%	16%	7%
Until the amelioration of the underlying disease	3%	5%	4%	–
Other	0%	0%	0%	4%

6. Empiric therapy for fever refractory to broad-spectrum antibacterials in neutropenic patients

Q.18 When selecting an antifungal drug as first choice for empiric therapy in patients with fever refractory to antibacterials, what features of the drug are considered of primary importance? (up to 2 answers)

	Group A	Group B	Group A+B	Historical Control
Antimycotic activity (MIC)	43%	44%	43%	11%
Pharmacokinetics	20%	17%	18%	4%
Safety	35%	37%	35%	28%
Compliance	2%	3%	3%	17%
Cost (economy)	0%	0%	0%	2%
Other	1%	0%	≤1%	4%
The drug assumed to work best on the presumed fungus	–	–	–	36%

Q.19 Which is the fungus that you first take into consideration for empiric therapy in patients with fever refractory to antibacterials?

	Group A	Group B	Group A+B	Historical Control
C. albicans	77%	80%	79%	52%
C. glabrata	0%	5%	2%	0%
C. parapsilosis	2%	3%	2%	0%
Other *Candida* species	3%	5%	4%	48%
Aspergillus species	13%	7%	10%	0%

Cryptococcus	2%	0%	1%	0%
Other eumycetes	3%	0%	2%	0%

Q.20 When beginning the administration of antifungal drugs for empiric therapy of patients with fever refractory to antibacterials, what is the drug selected as first choice?

	Group A	Group B	Group A+B	Historical Control
Injectable amphotericin B	2%	0%	1%	0%
Oral fluconazole	12%	2%	7%	9%
Injectable fluconazole (injectable fosfluconazole)	73%	93%	83%	89%
Oral itraconazole	2%	0%	1%	0%
Injectable miconazole	0%	0%	0%	2%
Oral flucytosine	0%	0%	0%	0%
Injectable micafungin	11%	5%	8%	–

Q.21 When injectable fluconazole (*including injectable fosfluconazole) is selected as the first choice for empiric therapy in patients with fever refractory to antibacterials, what is the daily maintenance dosage? (for adults)

	Group A*	Group B*	Group A*+B*	Historical Control
50 mg/day	0%	0%	0%	0%
100 mg/day	3%	7%	5%	7%
200 mg/day	64%	51%	58%	63%
400 mg/day	29%	42%	35%	30%
More than the above indicated dosages	0%	0%	0%	0%
It is not selected as first choice	4%	0%	2%	0%

7. Invasive pulmonary aspergillosis

Q.22 What are the criteria used to determine the start of empiric therapy in cases of presumed invasive pulmonary aspergillosis afflicting neutropenic patients? (up to 2 answers)

	Group A	Group B	Group A+B	Historical Control
Appearance of fever refractory to antibacterials	17%	13%	15%	
Abnormal shadow on chest radiography (regardless of fever)	19%	12%	15%	
Appearance of fever refractory to antibacterials + abnormal shadow on chest radiography	23%	17%	20%	No data available
Abnormal shadow on chest radiography + positive aspergillus antigen	19%	30%	25%	
Appearance of fever refractory to antibacterials + positive aspergillus antigen	16%	26%	21%	
Appearance of fever refractory to antibacterials + halo sign	5%	1%	3%	

Q.23 What kind of antifungal drug is used at the beginning of empiric therapy for invasive pulmonary aspergillosis afflicting neutropenic patients?

	Group A	Group B	Group A+B	Historical Control
Fluconazole (fosfluconazole)	7%	2%	5%	
Amphotericin B	37%	37%	36%	
Micafungin	36%	49%	42%	
Itraconazole capsules	6%	2%	4%	
Amphotericin B + azole	1%	2%	2%	No data available
Amphotericin B + micafungin	10%	2%	6%	
Micafungin + azole	3%	5%	4%	

8. On the guidelines for the diagnosis and treatment of invasive fungal infections

Q.24 Since the guidelines for the diagnosis and treatment of invasive fungal infections were released, have your diagnosis and treatment practices changed?

	Group A	Group B	Group A+B	Historical Control
They have changed considerably	11%	7%	9%	
They have somewhat changed	41%	54%	47%	No data available
They have changed just a little	31%	27%	29%	
They have not changed at all	17%	13%	15%	

Q.25 Which of the following items of the guidelines for the diagnosis and treatment of invasive fungal infections do you refer to?

	Group A	Group B	Group A+B	Historical Control
Diagnosis and treatment in all their aspects	27%	43%	34%	
Primarily the flowchart	28%	14%	21%	
Primarily the diagnosis sections	6%	5%	6%	
Primarily the sections on treatment	14%	18%	16%	No data available
The serological test and the information attached to the list of antifungals	19%	4%	11%	
No particular item	6%	16%	11%	

Q.26 What are your expectations relative to the future of the guidelines for the diagnosis and treatment of invasive fungal infections? (up to 2 answers)

	Group A	Group B	Group A+B	Historical Control
Concrete guidelines based on more evident proofs	32%	32%	32%	
More concise and compact guidelines	13%	14%	13%	
Enhancement of the explanatory parts	6%	8%	7%	
More concreteness in relation to diagnosis and treatment	14%	26%	20%	No data available
Greater speed in reviewing and revising the guidelines in relation to new diagnostic methods and the release of new antifungals	33%	20%	27%	
Other	2%	0%	1%	

9. Expectations relative to the antifungals of the future

Q.27 Which are the most important features in relation to future antifungals? (up to 2 answers)

	Group A	Group B	Group A+B	Historical Control
Enhanced antifungal activity against *Candida* spp.	5%	22%	13%	
Enhanced antifungal activity against *Aspergillus* spp.	34%	28%	31%	
Improvement in safety	28%	28%	28%	
Improvement in pharmacokinetics	10%	4%	7%	
Availability of both oral and injectable preparations	10%	10%	10%	No data available
Economical efficiency/benefits	13%	8%	10%	
Other	0%	0%	0%	

Q.28 In the years to come, which of the following future drugs would you define as having the highest expectations?

	Group A	Group B	Group A+B	Historical Control
Voriconazole (oral and injectable)	70%	61%	65%	
Injectable itraconazole	11%	11%	11%	
Itraconazole oral solution	0%	0%	0%	No data available
Lipid amphotericin B formulation (lipidization)	14%	17%	16%	
Other	5%	11%	7%	

Discussion

During the Interactive Session held at the 5th Annual Meeting of the Mycoses Forum, a questionnaire survey (Group A) was conducted regarding the "Guidelines for the Diagnosis and Treatment of Deep-Seated Mycosis" that had been released the previous year. The goals of the survey were to examine the extent to which the guidelines were used in the clinical field, whether they were easy for clinicians to use, and whether they needed modification.

The results were compared with the results of the similar survey conducted at the 72nd Annual Meeting (April 1998) of the Japanese Association for Infectious Diseases held before the guidelines were released (Historical Control). Additionally, the survey (Group B) conducted during the Oita Prefecture Deep-Seated Mycosis Workshop, held in March 2004 was cataloged as a reference. The participants' background showed a tendency toward a greater number of infectious disease specialists in Group A, general clinicians in Group B, and fungal infection specialists in the Historical Control.

Frequency of deep candidiasis

Among all infectious diseases, the overall percentage of deep candidiasis was 10% or less in most facilities. However, both in the previous and current survey, in some of the facilities, deep candidiasis accounted for 50% of the total;

thus, differences in the frequency of candidiasis appear to depend on the facility. As for the causative organisms, *C. albicans* accounted for the majority of cases in most facilities. However, in other specialties such as pediatrics, obstetrics and gynecology, ophthalmology, or infectious diseases, an increasing tendency toward non-*albicans Candida* species other than *C. albicans* is observed.

In a survey conducted in 1999 of surgery and critical care patients with fungal infections, to whom antifungals were administered, the blood isolates in 8 out of 12 candidemia patients were *C. albicans*.[8] The results of another survey carried out in 156 facilities across the country in 2001–2002[9] showed an increase in non-*albicans Candida*, with *C. albicans* accounting for 40.7% of cases, and *C. parapsilosis* and *C. glabrata* accounting for 23.0% and 17.9% of cases, respectively. In the current survey, there was a higher proportion of cases due to *C. glabrata* than *C. parapsilosis* – the answer for *C. albicans* and *C. glabrata* was 18%, and for *C. albicans* and *C. parapsilosis* 6%. This result might be due to the difference between surveillance carried out in large-scale hospitals such as university hospitals where there are many serious and persistent cases, and our survey in which many clinicians participated.

Diagnosis of invasive fungal infections

Generally, when a patient does not respond to antibacterials, the possibility of fungal infection is considered. Depending on the persistence of fever after the use of antibacterials, the beginning of antifungal administration occurred most frequently after 5 days (31% of answers) or 7 days (30% of answers). In the previous survey, antifungal therapy occurred most frequently after 3 days (40% of answers). Therefore, there was a trend toward a later initiation of antifungal therapy in the current survey. This difference might be explained by the practice that diagnostic procedures are instituted according to the guidelines in order to begin the treatment after determining the key features of the fungal infection.

For the diagnosis of candidiasis, serological tests (43% of respondents) and blood culture (40%) were the most common methods. In the previous survey, serological tests were used by 72% and blood culture by only 5% of respondents, confirming a decreased use of serological tests in recent years. This finding might be due to the fact that, during the earlier survey, serological tests were a new technique, but after widely used in clinical practice, serological tests were shown to be less specific as the sole diagnostic method. At present, along with serological tests, a number of other tests are done at the same time, corresponding to the guidelines flowchart, thus confirming a positive trend. Furthermore, as the Candi-tec antigen test conducted in the past has low reliability,[10] and as serological tests offers comparatively better sensitivity and specificity based on the beta-D-glucan value,[11] such a method also is recommended in the guidelines.

Survey answers showed the importance of surveillance culture in cases with positive serological tests; the choice stating that in these cases, therapy is initiated even with only one positive site at surveillance culture was the most selected one. On the other hand, 36% of respondents begin treatment in cases with negative serological tests and two or more sites positive at surveillance culture, showing that in many facilities treatment is initiated when there are many positive sites on surveillance culture. Those cases that present many positive sites at surveillance culture are prone to a higher incidence of progression to candidemia;[12,13] therefore, the guidelines recommend surveillance culture as one criterion for initiating treatment. The results of this survey reflect the guidelines recommendations. Finally, at the Oita Workshop many participants were strongly in favor of not taking into consideration the surveillance culture results. This result might be simply due to the fact that many non-expert members who lack enough understanding of surveillance culture participated in the workshop held in a local city.

Antifungal treatment of candidemia

In cases where *C. albicans* is identified in blood culture, injectable fluconazole was selected as first-choice therapy most often both in the previous and current survey. However, in the earlier survey, 93% chose injectable fluconazole compared to 81% chose fluconazole or fosfluconazole in the current survey, reflecting increased use of oral fluconazole and the newly introduced Micafungin. As expected, injectable fluconazole still is considered the first choice par excellence because of its successful results in the treatment of *C. albicans*.

In patients with *C. albicans* candidemia treated with injectable fluconazole (or fosfluconazole), 53% of the facilities used a maintenance dosage of 400 mg per day; these results were similar to those of the previous survey. Respondents who use a 200-mg dose accounted for 40% of the total. However, the guidelines recommend the 400-mg dosage of fluconazole for candidemia patients because the higher dosage has been shown to be more effective in these cases.[14] In the survey conducted at the Oita Prefecture Workshop, many respondents use a 50-mg maintenance dosage of Micafungin. Again, this finding might be explained by the inclusion of non-experts with a poor understanding of dosages.

In fungal infections caused by *C. parapsilosis*, the first choice was, as expected, injectable fluconazole (or fosfluconazole), which accounted for 48% of the answers. This choice was followed by Micafungin, which was chosen by 31% of respondents, and by Amphotericin, which accounted for 10% of the answers. Compared with the previous survey, fluconazole (or fosfluconazole), which previously had dominated the scene, had to share the field with other drugs. Because the minimum inhibitory concentration (MIC) of Micafungin is not considered suitable for treating *C. parapsilosis*, once again the first choice par excellence remains fluconazole or fosfluconazole. Furthermore, in the survey conducted at the Oita Workshop, the majority of respondents chose fluconazole as the first choice

for treatment of both *C. albicans* and *C. parapsilosis*. The fact that many doctors involved in surgery took part at the Oita Workshop may have had a certain influence on the results.

On the other hand, because Micafungin is known to be very effective for the treatment of *C. glabrata*, its selection as first choice is valid. However, at the Oita Workshop there was evidence of a tendency toward a poor understanding of the effectiveness and appropriate dosages of Micafungin.

Completion of antifungal therapy against candidemia and disseminated candidiasis

As an indicator of when to stop administering antifungal therapy for candidiasis associated with amelioration of the underlying disease, in the earlier survey, clinical symptoms accounted for 85% of the answers, while in the current survey, clinical symptoms accounted for only 50%. In the current survey, serological test accounted for 25% and imaging diagnosis accounted for 14% of answers. Even at the Oita Workshop the answers were divided between clinical symptoms and serological test, and the latter choice was chosen as the goal for the complication of drug administration. Certainly a serological test can be considered an excellent objective criterion, but as neither serological test nor imaging diagnosis can rapidly reflect the improvement by antifungal therapy, there is a considerable risk that treatment may be prolonged unnecessarily when using such a criterion. The serological test may be only useful in establishing a diagnosis, but for assessment of the complications of antifungal therapy, clinical signs and symptoms are more meaningful than serological tests.

For the question regarding the period for assessment of candidiasis treatment, the most common answer was 1 week (35%), followed by 3 days (14%). The results at the Oita Workshop were similar. In case of antibacterials, the goal is usually achieved after 3 days with declining of fever and the consequent termination of drug administration, but in the case of antifungals there is no such goal at present, so the answers were varied. However, the manifestation of therapeutic results in candidiasis occurs late in the course of treatment, and fluconazole takes approximately 7 days to achieve a sufficient blood concentration.[15] Thus, it is advisable to avoid interruption of therapy after 3 days because appreciable results are lacking. It is recommended that therapy be continued for 7 days before assessment of results.

Empiric therapy for fever refractory to broad-spectrum antibacterials in neutropenic patients

The most important factors in choosing antifungal therapy for patients with fever refractory to antibacterials were indicated as MIC in 43% of the answers, followed by safety and pharmacokinetics. In the azole family of antifungals, the antimicrobial action was confirmed to be dependent on MIC and area under the curve,[16] and penetration to tissues also is a critical factor. Consequently, the answers are reasonable.

In many specialties, *C. albicans* was the most selected first fungi to be taken into consideration for the empiric therapy of patients with fever refractory to antibacterials. In these cases, injectable fluconazole was selected as the drug to be administered. The majority of respondents in the current survey use a 200 mg maintenance dosage of fluconazole (64%); a similar result was found in the other surveys. The dosage for target therapy recommended in the guidelines ranges from 200 to 400 mg; therefore, even though it might produce disappointing results, 200 mg can be considered a valid dosage.

Influence of the guidelines for the diagnosis and treatment of invasive fungal infections

The survey was instituted to determine to what extent the guidelines have been understood and accepted by clinicians. First, participants were asked whether a change had taken place compared to the past in diagnosis and treatment according to the guidelines. The most common answer was "They have somewhat changed," accounting for 41% of the total, followed by the answer "They have changed just a little." The results from the Oita Workshop were similar. In combination, the answers stating that there was no particular change or no change at all accounted for approximately 50% of the total, leading one to think that in the future it will be necessary to further revise the guidelines.

In regard to how the guidelines are used, the flowcharts are often used. In the present guidelines, recommendations were simplified by creating flowcharts for each section. The fact that the flowcharts gathered satisfactory consensus and appreciation is a positive reflection on the guidelines.

When participants were asked about their expectations for future guidelines, a desire for concrete matters based on more evident proofs and a desire for revisions to reflect new diagnostic methods and the release of new antifungals were chosen by an almost equal percentage of respondents. In terms of evidence level, the same ranking method used in foreign guidelines should seriously be taken into consideration. Participants also expressed a desire for clearer explanations about the features and appropriate use of the new drugs that are expected to be introduced to the clinical field.

Expectations of newer antifungals

In relation to future antifungals, new drugs effective against *Aspergillus* species are eagerly awaited. Both surveys showed that in the years to come, the greatest expectations for new drugs focused on voriconazole and, as expected, once again the question of treating infections caused by the genus *Aspergillus* remains a key issue.

References

1. Clark TA, Hajjeh RA. Recent trends in the epidemiology of invasive mycoses. Curr Opin Infect Dis 2002;15:569–74.
2. Kim K, Lee MH, Kim J, Lee KS, Kim SM, Jung MP, et al. Importance of open lung biopsy in the diagnosis of invasive pulmonary aspergillosis in patients with hematologic malignancies. Am J Hematol 2002;71:75–9.
3. Abi-Said D, Anaissie E, Uzun O, Raad I, Pinzcowski H, Vartivarian S. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin Infect Dis 1997;24:1122–8.
4. Kume H, Abe M. The epidemiology of pulmonary mycosis (In Japanese). Clin Microorg 2000;27:133–9.
5. The Infectious Diseases Society of America (IDSA). IDSA guidelines for management of systemic fungal infection. Clin Infect Dis 2000;30:652–718.
6. European Organization for Research on Treatment of Cancer (EORTC). Guidelines for diagnosis of invasive fungal infections. Available at: http://www.aspergillus.man.ac.uk.
7. Deep-seated Mycosis Guidelines Editorial Committee. Guidelines for the diagnosis and the treatment of deep-seated mycosis. (In Japanese). Tokyo: Ishiyaku; 2003.
8. Aikawa N, Sumiyama Y, Kusachi S, Hirasawa H, Oda S, Yamazaki Y. Use of antifungal agents in febrile patients non-responsive to antibacterial treatment: the current status in surgical and critical care patients in Japan. J Infect Chemother 2002;8:237–41.
9. Takakura S, Fujihara N, Saito T, Kudo T, Iinuma Y, Ichiyama S. National Surveillance of species distribution in blood isolates of *Candida* species in Japan and their susceptibility to six antifungal agents including voriconazole and micafungin. J Antimicrob Chemother 2004;53:283–9.
10. Phillips P, Dowd A, Jewesson P, Radigan G, Tweeddale MG, Clarke A, et al. Nonvalue of antigen detection immunoassays for diagnosis of candidemia. J Clin Microbiol 1990;28:2320–6.
11. Obayashi T, Yoshida M, Mori T, Goto H, Yasuoka A, Iwasaki H, et al. Plasma (1,3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 1995;345:17–20.
12. Martino P, Girmenia C, Micozzi A, Raccah R, Gentile G, Venditti M, et al. Fungemia in patients with leukemia. Am J Med Sci 1993;306:225–32.
13. Rangel-Frausto MS, Wiblin T, Blumberg HM, Saiman L, Patterson J, Rinaldi M, et al. National Epidemiology of Mycoses Survey (NEMIS): Variations in rates of bloodstream infections due to *Candida* species in seven surgical intensive care units and six neonatal intensive care units. Clin Infect Dis 1999;29:253–8.
14. Graninger W, Presteril E, Schneeweiss B, Teleky B, Georgopoulos A. Treatment of *Candida albicans* fungaemia with fluconazole. J Infect 1993;26:133–46.
15. Nakashima M. The clinical study of fluconazole against pulmonary mycosis. Effects of fluconazole on pulmonary cryptococcosis and aspergillosis, and its pharmacokinetics in patients (in Japanese). Jpn J Antibiot 1989;42:127–37.
16. Louie A, Drusano GL, Banerjee P, Liu QF, Liu W, Kaw P, et al. Pharmacodynamics of fluconazole in a murine model of systemic candidiasis. Antimicrob Agents Chemother 1998;42:1105–9.

Flow-Independent Myocardial Ischemia Induced by Endothelin-1

An NADH Fluorescence Analysis

Soushin Inoue, MD, Shingo Hori, MD,† Takeshi Adachi, MD,‡ Koji Miyazaki, MD,* Shingo Kyotani, MD,§ Keiichi Fukuda, MD,* Hidezo Mori, MD,§ Hiroe Nakazawa, MD,¶ Naoki Aikawa, MD,† and Satoshi Ogawa, MD**

Abstract: The endothelin-1 (ET-1) is known to cause myocardial ischemia; however, whether this effect is entirely dependent on vasoconstriction is uncertain. The aim of this study was to characterize the myocardial ischemia after the intracoronary administration of endothelin-1, and compare it with that induced by coronary stenosis. In the left anterior descending coronary artery of 15 dogs, a mild inflow reduction (30%) was produced for 10 minutes using intracoronary endothelin-1 (46 ± 33 pmol/min) or coronary stenosis. The hearts were rapidly cross-sectioned at short axial plane and freeze-clamped within 120 milliseconds using a specially developed device to visualize and quantify the area of ischemia (%IA) with NADH fluorescence photography. The %IA was larger in the endothelin-1 group than in the stenosis group (66 ± 23 versus 18 ± 18, $P = 0.0005$); furthermore, the ischemia was transmural in the ET-1 group, but limited to subendocardium in the stenosis group. ET-1 increased the coronary arterial resistance especially in subepicardial region and produced smaller ischemic foci in microcirculation. The mechanism of larger ischemia produced by ET-1 might depend on pro-ischemic effects on myocytes and vasoconstriction of the coronary microcirculation, predominantly in the subepicardium in vivo.

Key Words: coronary circulation, endothelin-1, microcirculation, myocardial ischemia, NADH fluorescence, pro-ischemia

(*J Cardiovasc Pharmacol*™ 2005;46:810–816)

Endothelin-1 (ET-1) is the most potent vasoactive peptide derived from the endothelium[1] and constricts vascular smooth muscles to induce hypoperfusion.[2] The plasma ET-1 was found to be increased in patients with acute myocardial infarction and angina pectoris.[3] The pathophysiological role of ET-1 for ischemic heart disease is relevant but not fully clarified yet. Others and we have previously reported that the intracoronary administration of ET-1 induces a prominent coronary flow reduction, ST elevation, apical systolic bulge, and impaired left ventricular diastolic function in vivo.[4–6] The reduced cardiac function with ST-elevation indicated that ET-1 induces myocardial ischemia by decreasing coronary flow. However, the systolic bulge induced by ET-1 was unexpected and could not be explained by standard criteria for myocardial ischemia using a reduced flow model, which predominantly decreases the subendocardial blood flow.[4] Moreover, ET-1 receptor antagonists were shown to reduce the myocardial infarction size in ischemia/reperfusion models without any changes in regional myocardial blood flow, suggesting that intrinsic ET-1 has a flow-independent effect on myocardial ischemia in vivo.[7,8] In addition to its vasoconstrictive effect, ET-1 has direct effects on cardiac myocytes, including positive inotropic, chronotropic, and metabolic effects.[9–12] Therefore, myocardial ischemic metabolism and flow distribution should be compared in ET-1 administration and simple flow-reduction models.

The aim of this study was to clarify the characteristics of myocardial ischemia induced by the intracoronary administration of ET-1 under condition of controlled mild hypoperfusion in a canine model. The extension, distribution, and severity of myocardial ischemia induced by ET-1 were compared with those induced by an equivalent coronary flow reduction introduced by occluding the bypass circuit to the coronary artery. Because the severity and the distribution of metabolic changes in ET-1–induced myocardial ischemia were not quantified in previous reports, the flow-dependent and -independent pro-ischemic effects of ET-1 have not been sufficiently differentiated. In this study, we applied an NADH fluorescence method to rapidly cross-sectioned frozen heart slices, because NADH is a sensitive marker of ischemia and the distribution of the myocardial ischemic region can be clearly visualized by UV fluorescence. Regional metabolite sampling from frozen heart tissue also reveals the distribution and severity of ischemic myocardium.[13,14] This specially developed sampling method has been shown to be rapid enough to preserve the redox states that existed prior to sectioning, because the sectioning and freezing is performed within 120 milliseconds.[13]

Received for publication April 29, 2005; accepted September 12, 2005.

From the *Cardiopulmonary Division, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; †Department of Emergency and Critical Care Medicine, Keio University School of Medicine, Tokyo, Japan; ‡Department of Biochemistry and Integrative Medical Biology, Keio University School of Medicine, Tokyo, Japan; §National Cardiovascular Center; and ¶Department of Physiology, Tokai University School of Medicine.

Reprints: Shingo Hori, Department of Emergency Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582 Japan (e-mail: shingo@sc.itc.keio.ac.jp).

METHODS

The present investigation conformed with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Animal Preparation

Mongrel dogs weighing 9 to 12 kg (n = 22) were anesthetized with pentobarbital (35 mg/kg iv) and sustained under positive-pressure ventilation. A bilateral thoracotomy was performed at the sixth intercostal space, and the heart was suspended in a pericardial cradle. Heparin was administered intravenously at 500 units/kg. To measure the aortic pressure, a 7F catheter was introduced from the femoral artery into the ascending aorta. A bypass made of silicon tube with metal tip was cannulated from a left subclavian artery to the left anterior descending coronary artery (LAD) on beating heart. The proximal LAD was ligated and cut down to insert a metal tip of the bypass. The diameter of left subclavian artery may not be suitable for coronary diameter but is applicable for bypass-supplier because of avoiding operative failure and excessive bleeding on preparation. A pressure transducer (TP400P, Nihon Koden, Co. Ltd., Tokyo, Japan) and an electromagnetic flow probe (MFV-1200, Nihon Koden, Co. Ltd.) were installed on the bypass circuit, and the coronary perfusion pressure and coronary blood flow were continuously monitored and recorded at thermograph. To record the epicardial electrocardiogram, an electrode made of Ag-AgCl was placed at the center of the perfused area of the left anterior descending coronary artery. To administer the fluorescent dye or non-radioactive microspheres, a 7F catheter was inserted into the left cardiac auricle. The heart rate was fixed at 200 beats per min (bpm) using left atrial pacing. Before starting the experimental protocol, the bypass circuit was occluded for 15 seconds and dogs with reactive hyperemia below 200% of the control flow level were excluded from the study.

Protocol 1: Visualization of Tissue Redox State and Chemical Analysis of Myocardial Ischemia

Fifteen dogs were randomly divided into two groups; eight received the ET-1 administration (endothelin group) and seven underwent coronary stenosis (stenosis group). In the endothelin group, ET-1 (Peptide Institute Inc., Osaka, Japan; 10–150 pmol/min) was administered continuously into the silicon bypass circuit using a micro-infusion pump[15]: this dose reduced the coronary flow by 20% to 40% of the control level. The dosage of ET-1 rapidly increased within 1 minute to get the target flow reduction. The same speed of ET-1 injection could keep in steady state flow reduction, but some dogs with reduced flow level over 40% were excluded from the study. In the stenosis group, coronary inflow was reduced to a comparable level (20%–40% of the control level) by mechanical constriction of the bypass tube. The hemodynamic parameters and epicardial electrocardiogram (ECG) were recorded every minute. The ST segment of the epicardial ECG was measured at 100 milliseconds from the initiation of the QRS complex. At 10 minutes after coronary flow reduction, the beating heart was rapidly cross-sectioned into 4-mm-thick slices along the short-axis plane of the left ventricle using a sampling device specially developed by Hori et al.[13] Within 120 milliseconds after cross-sectioning, a cross-sectional heart slice was compressed to 2.4-mm thickness using precooled aluminum blocks at −190°C. To visualize the tissue redox state of the bypass-perfused areas, from where the cross-sectioned heart slices were obtained, 10 mL of fluorescent dye solution (rhodamine B fluid: 0.1 mg/mL/kg) was injected into the left cardiac auricle 5 seconds before cross-sectioning. The frozen heart slices were fixed in a specially made container filled with precooled freon-12 (CCl_2F_2) and preserved in liquid nitrogen.

Visualization of Tissue Redox State by NADH Fluorescence Imaging

The increase in NADH is the most sensitive and rapid intracellular reaction to occur during ischemia[16,17] and can be visualized as an increase in the NADH fluorescence level in ischemic myocardium. Dual fluorescence photography of NADH (indicating ischemia) and rhodamine B (indicating bypass non-perfused areas) was applied to 15 frozen heart slices. A pair of excitation lights (360 nm, Model B-100A, Ultra-Violet Products, CA) was applied to the frozen heart slices at liquid-nitrogen temperature to visualize the surface NADH and rhodamine B fluorescence in the cross-sectional plane. Two band pass filters were used as secondary filters (Kodak Wratten Gelatin Filter 2E and 47 (Kodak Japan Co. Ltd., Tokyo, Japan) for NADH fluorescence, Kenko Optical Filter YA-3 (Kenko Co. Ltd, Tokyo, Japan) for rhodamine B fluorescence). To quantify the area of myocardial ischemia, the surface NADH fluorescence was analyzed using an image analyzer (IBAS, Carl Zeiss Japan Co. Ltd., Tokyo, Japan) via a CCD video camera (XC-77, Sony Japan Co. Ltd., Tokyo, Japan). A positive NADH-fluorescent area (ischemic area) was defined as an area with a fluorescence intensity two standard deviations above the mean of the simultaneously measured control frozen slice, given another non-ischemic canine cross-sectional heart sample. The bypass-perfused area was defined as the rhodamine B fluorescence-negative area. In a previous study, we observed that rhodamine B, injected into the left cardiac auricle before 5 minutes of cross-sectioning, was not identified in bypass-perfused areas and was clearly present in non-perfused areas.[14] To quantify the amount of ischemic myocardium in the heart slices, the extent of ischemia was expressed as a percentage of the NADH-fluorescent area to the bypass-perfused area in the subendocardial and subepicardial halves of the frozen slices.

To visualize the fluorescent area at a high resolution of up to 10 micrometers, magnified NADH fluorescence photography (×100) was also applied using a dissecting microscope and a Xenon excitation light (Supercure-201S, Fibernics Co. Ltd., Saitama, Japan). To measure the area of microischemia, ischemic foci at the microcirculatory level were selected at random in the endothelin group (1191 counts from 8 randomly selected hearts) and the stenosis group (703 counts from 7 randomly selected hearts). An image analyzer (IBAS, Carl Zeiss Japan Co. Ltd.) was used to measure their short-axis diameters.

Chemical Analysis for Myocardial Metabolites

Cylindrical microsamples weighing about 10 mg (2.4 mm in diameter and 2.4 mm in depth) were drilled in the liquid-nitrogen frozen slices; the microsamples were obtained in perfused area of the subendocardium or subepicardium exhibiting NADH fluorescence. NADH fluorescence was used as a guide to select the sampling sites. Four to six microsamples from each heart slice, for a total of 62 microsamples, were obtained. The NAD, NADH, ATP, creatine phosphate (CP), and lactate concentrations were analyzed in each microsample. NAD and NADH were measured using the bacterial luciferase method,[18] ATP and creatine phosphate were measured using the luciferin-luciferase method,[13] and lactate was measured using the LDH method.[19] The protein content was determined using the Lowry method.[20]

Protocol 2: Systemic and Coronary Hemodynamics

To measure myocardial tissue flow without affecting myocardial ischemia in the same animal, coronary flow was successively reduced in an additional seven dogs, first by stenosis and then by ET-1. After performing a baseline measurement (control group), the bypass was constricted and tissue flow was measured after 10 minutes (stenosis group). The stenosis was then released and once the hyperemia had stabilized, ET-1 (10–150 pmol/min) was administered intracoronary to induce the same degree of hypoperfusion and tissue flow was measured again after 10 minutes (endothelin group). Because of the sustained vasoconstriction induced by ET-1, the order of hypoperfusion induced was fixed, not random.

Myocardial tissue flow was measured using nonradioactive microspheres (15 micrometers in diameter), made of inert plastic labeled with stable heavy elements (Sekisui Plastic Co. Ltd., Tokyo, Japan).[21] Microspheres labeled with barium, iodide, zirconium, or bromine were suspended in 0.1% sodium dodecyl sulfate (SDS) solution, and $0.5–1.0 \times 10^7$ microspheres were infused into the left cardiac auricle. The microspheres were well shaken, and mixed mechanically in a syringe prior to or during the protocol, and no aggregations were observed using light microscopy. Heart tissue samples weighing 3 to 5 g were taken from the subendocardial and subepicardial layers of the bypass-perfused area. Tissues and reference arterial blood samples were dissolved in 1N-KOH, and the microspheres were trapped on filter papers. The samples were irradiated with X-rays, and the X-ray fluorescence activity of each heavy element was measured, the amount of microspheres and the tissue flow in each sample were then calculated.

Statistical Analysis

All values were presented as the means ± SD. Changes in tissue flow and hemodynamic data were determined using a two-way ANOVA. For paired or unpaired data between two groups, Student *t* test was used. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Protocol 1

Hemodynamics and Epicardial Echocardiography

The mean aortic pressures before the experimental protocol were not significantly different between the endothelin group and the stenosis group (Table 1). The hemodynamics did not change significantly throughout the experimental period. The mean doses of endothelin-1 were 46 ± 33 pmol/min. No significant difference in coronary flow reduction was seen in either the endothelin group or the stenosis group. In contrast, the changes in the epicardial ST segment were significantly larger in the endothelin group than in the stenosis group. These results suggest that the myocardial ischemia caused by endothelin-1 differed from that caused by coronary stenosis.

Extension and Distribution of NADH Fluorescence

Figure 1 shows NADH fluorescence photographs of frozen heart slices from both groups. Myocardial ischemia was transmurally distributed in the endothelin group, whereas ischemia distribution was limited to the subendocardium in the stenosis group. Consequently, the total area of myocardial ischemia was larger in the endothelin group than in the stenosis group (Table 2).

Magnified fluorescence photography (×100) was applied to evaluate ischemia formation at the microcirculatory level. Microischemic spots, round or rectangular in shape depending on the direction of the myocardial fibers, were observed in both groups (Fig. 2, upper photographs). To measure the size of the ischemic spots, the short-axis length of the spot was calculated (Fig. 2, lower bar graph). The mean value of the short-axis diameter (less than 120 micrometers) was significantly smaller in the endothelin group than in the stenosis group (61.1 ± 18 versus 76.4 ± 18 micrometers; $P < 0.0001$).

TABLE 1. Changes in ST Segment and Hemodynamics

	Endothelin Group (n = 8) Mean (SD)	Stenosis Group (n = 7) Mean (SD)	Statistics
Changes in ST segment (mV)	6.5 (4.5)	0.7 (0.5)	$P = 0.006$
Coronary blood flow reduction (%)	32.3 (4.5)	34.0 (4.2)	ns
Heart rate (/min)	186 (20)	200 (3.3)	ns
Mean aortic pressure (mm Hg)	99 (8.7)	109 (12)	ns
Mean coronary perfusion pressure (mm Hg)	99 (8.3)	64 (12)	$P < 0.0001$

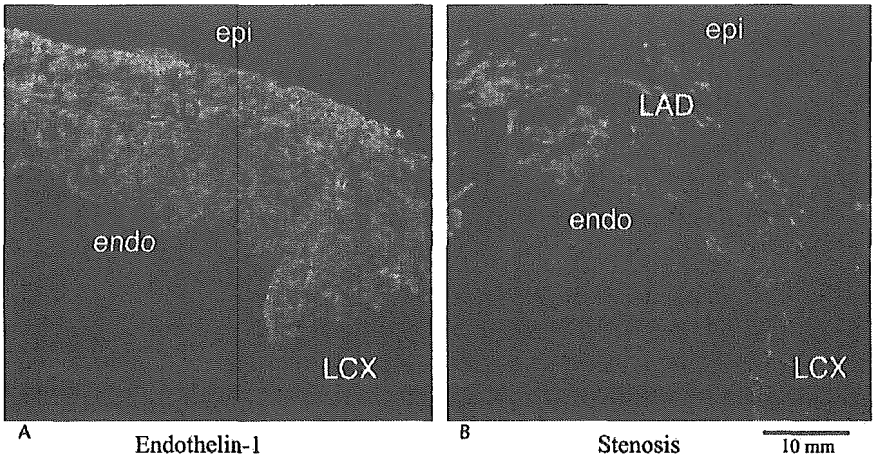


FIGURE 1. NADH fluorescence photographs of cross-sectional frozen heart slices. The slices show short-axial view at mid-left ventricle. LAD, left anterior descending coronary artery perfused myocardium; LCX, left circumflex coronary artery perfused myocardium; Epi, Epicardial side; Endo, Endocardial side. A, Endothelin-1 decreased coronary inflow by 36% and produced a transmural extension of positive NADH fluorescence. B, Stenosis reduced coronary inflow by 32% and produced positive NADH fluorescence (ischemia) in the subendocardium.

Chemical Analyses of Tissue Metabolites

The higher NADH content as well as the lower NAD/NADH ratio in both the subepicardial and the subendocardial layers of the ischemic LAD areas in the endothelin group indicated more severe anaerobic metabolism than in the stenosis group (Table 3). These indicators are the most sensitive marker of the redox states in ischemic myocardium; no difference in classic ischemic markers like lactate, ATP, or CP were observed between the two treatment groups (Table 3).

In NADH-fluorescent myocardium, the NADH concentrations were significantly higher than those in NADH-non–fluorescent areas (0.77 ± 0.20 versus 0.40 ± 0.08 nmol/mg, n = 27, and 33, respectively, $P < 0.0001$). This result confirmed the strong link between positive NADH fluorescence and the anaerobic state in frozen heart samples.

Protocol 2

Regional Coronary Blood Flow

In a preliminary study, the injection of microspheres (more than 1×10^4/g) into canine hearts to measure myocardial flow was shown to produce microspots of NADH fluorescence caused by microsphere embolization-induced ischemia,[22] indicating that the concomitant measurement of tissue flow in the hearts of animals treated with protocol 1 could not be accurately evaluated (Table 4). Therefore, we performed a separate but parallel protocol of NADH fluorescence study to analyze myocardial tissue flow using the microsphere method. The heart rates and mean aortic pressures were comparable with those in protocol 1 and did not change significantly throughout the experiment. Coronary inflow reductions were also comparable with those in protocol 1 (29.4 ± 5.2% in the endothelin group, and 30.0 ± 2.7% in the stenosis group, ns). Flow in myocardial tissue and the flow ratio in endo/epi tissues were not significantly reduced in either group, although the calculated subepicardial coronary vascular resistance was higher in the endothelin group than in the stenosis group. Neither the absolute flow value nor the endo/epi flow ratio were able to account for the larger ischemic areas observed in the endothelin group in protocol 1.

DISCUSSION

Endothelin-1 directly constricts coronary smooth muscle and decreases blood flow, leading to severe myocardial ischemia; however the effects of ET-1 on myocardial ischemia in vivo have not been well characterized. This study produced the following findings: (1) ET-1 enlarged the myocardial ischemic area detected by NADH fluorescence, compared with that induced by similar reduction in coronary flow arising from stenosis. Moreover, ET-1 decreased the NAD/NADH ratio in both the subendocardium and the subepicardium, compared with the situation in the stenosis group, suggesting that ET-1 has flow-independent, direct pro-ischemic effects on myocytes in vivo. (2) ET-1 increased the coronary vascular resistance dominantly in the subepicardium and increased the ischemic area and the NADH content in the same region. These data suggest that ET-1 more selectively constricts subepicardial arterioles, extends myocardial ischemia transmurally, and causes an ST-elevation. (3) ET-1 generated smaller ischemic foci in the microcirculation than those generated by stenosis,

TABLE 2. Quantification of Ischemic Area and Transmural Distribution

	Endothelin Group (n = 8)			Stenosis Group (n = 7)			Endothelin vs. Stenosis	
	Endo Mean (SD)	Epi Mean (SD)	Statistics	Endo Mean (SD)	Epi Mean (SD)	Statistics	Endo	Epi
Ischemic area								
Endo or epi (%)	66.3 (22.3)	66.8 (23.3)	ns	27.7 (27.2)	8.5 (10.3)	$P = 0.03$	$P = 0.01$	$P < 0.0001$
Total (%)	66.4 (22.5)			17.6 (18.2)			$P = 0.0005$	

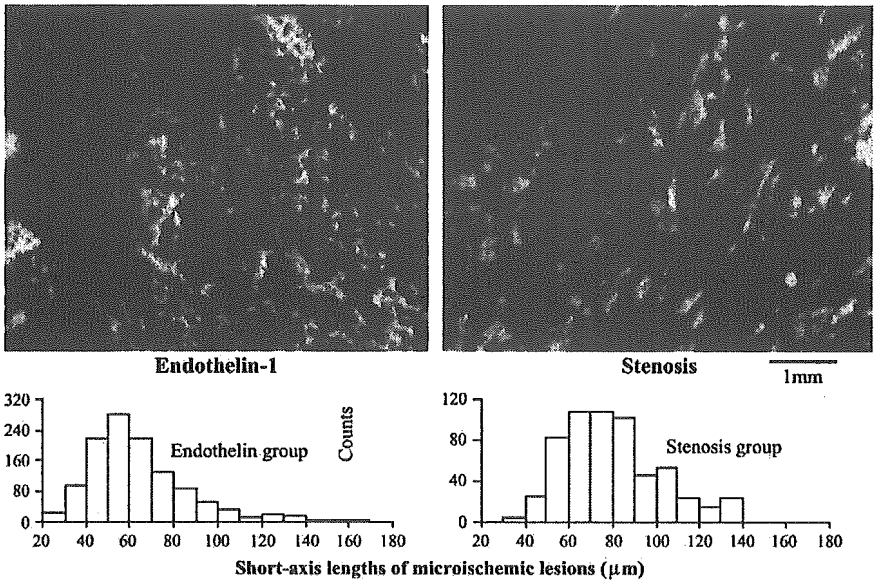


FIGURE 2. Magnified NADH fluorescence photographs (×100) showing microischemic foci in both the endothelin and stenosis groups. The histogram shows the distributions of the short-axis lengths of microischemic lesions in both groups. The endothelin group produced many similar shaped but smaller ischemic foci, compared with the stenosis group (61 ± 18 versus 76 ± 18 μm, $P < 0.0001$).

suggesting that the distal arteriolar levels of the coronary microcirculation may be affected by ET-1.

We found a clear dissociation between the reduction of coronary blood flow and the development of myocardial ischemia in the endothelin group, compared with the situation in the stenosis group. This finding was especially noticeable in the subendocardial region. In the endothelin group, we observed a subendocardial blood flow similar to that in the stenosis group, as detected using the non-radioisotope microsphere method. However, the ischemic area detected by NADH fluorescent was larger by more than two fold. This data clearly shows the flow-independent pro-ischemic effects of ET-1 in vivo. Although we did not clarify the direct mechanism of the pro-ischemic effects of ET-1 on myocytes, ET-1 may increase the oxygen demand of the myocytes. Previous reports have shown that endothelin-1 can increase intracellular Ca^{2+} through the activation of voltage-dependent Ca^{2+} currents[23,24] and can activate Na^{+}-H^{+} exchange on isolated myocytes,[25] both of which produce inotropic effects and augment oxygen demand.[10,11] An increased left ventricular dP/dt following the intravenous administration of endothelin-1 was also reported in canine and rat model.[9,26] Furthermore, using an ex vivo Langendorff model of rat hearts, Grover et al[27] demonstrated the extension of myocardial ischemia in arrested heart, suggesting the pro-ischemic effect of endothelin-1. ET-1 was also reported to augment myocardial mitochondrial damage induced by rotenone; therefore, mitochondrial dysfunction during ischemia may also be enhanced by ET-1.[28] ET-1 can elicit the positive-inotropic effects by the cross-talk with norepinephrine and a release of norepinephrine in the operation may enhance the pro-ischemic effects of ET-1.[29] In this study, ET-1 did not change the heart rate or the myocardial ATP content; however, we speculate that positive inotropic effects associated with an enhanced metabolic demand or an aggravation of mitochondrial dysfunction may cause the pro-ischemic effects of ET-1.

Another interesting finding was the difference in the distribution of myocardial ischemia between the endothelin and stenosis groups. In the coronary stenosis group, both the reduction of tissue flow and the distribution of ischemia were

TABLE 3. Chemical Analyses of Myocardial Microsampling of Subendocardial or Subepicardial Layer in the Endothelin and Stenosis Groups

	Endothelin Group			Stenosis Group			Endothelin vs. Stenosis	
	Endo Mean (SD)	Epi Mean (SD)	Statistics	Endo Mean (SD)	Epi Mean (SD)	Statistics	Endo	Epi
	n = 17	n = 17		n = 14	n = 14			
NADH (nmol/mg)	0.68 (0.25)	0.67 (0.27)	ns	0.5 (0.22)	0.39 (0.08)	ns	$P = 0.048$	$P = 0.001$
NAD/NADH	7.4 (2.9)	7.6 (3.5)	ns	10.9 (5.0)	12.5 (2.6)	ns	$P = 0.02$	$P = 0.0001$
Lactate (nmol/mg)	26.0 (12.9)	28.8 (13.9)	ns	31.0 (10.5)	22.9 (8.5)	$P = 0.04$	ns	ns
ATP (nmol/mg)	42.8 (8.5)	45.1 (11.1)	ns	43.0 (6.9)	41.7 (9.2)	ns	ns	ns
CP (nmol/mg)	67.3 (27.7)	74.7 (30.9)	ns	63.4 (11.7)	72.5 (20.3)	ns	ns	ns

ATP, adenosine triphosphate; CP, creatine phosphate.

TABLE 4. Tissue Flow and Hemodynamics in the Stenosis and Endothelin Groups

	Control Group Mean (SD)	Stenosis Group Mean (SD)	Endothelin Group Mean (SD)	ANOVA
CVR (mm Hg/mL/min/g)				
Endo	134 (48)	100 (35)	161 (90)	ns
Epi	118 (56)	67* (16)	160* (86)	$P = 0.03$
Tissue flow (mL/min/g)				
Endo	0.93 (0.29)	0.71 (0.23)	0.97 (0.53)	ns
Epi	1.14 (0.43)	1.01 (0.27)	0.86 (0.31)	ns
Heart rate (/min)	180 (13)	180 (12)	181 (13)	Ns
mAOP (mm Hg)	115 (15)	115 (15)	117 (14)	Ns
mCPP (mm Hg)	117† (13)	65†‡ (13)	119‡ (15)	$P < 0.0001$

CVR, coronary vascular resistance; mAOP, mean aortic pressure; mCPP, mean coronary perfusion pressure (n = 7).
*Stenosis group vs. endothelin group: $P = 0.0093$; †Control group vs. stenosis group: $P < 0.0001$; ‡Stenosis group vs. endothelin group: $P < 0.0001$.

predominant in the subendocardium. ET-1 administration, however, significantly increased the subepicardial coronary vascular resistance. The increase in the ST-elevation by ET-1 could be due to the transmural development of myocardial ischemia, because subepicardial ischemia widens the solid angle of the subepicardial electrode toward the ischemia front, resulting in an increase in the ST-segment.[30] A decrease in subepicardial blood flow induced by ET-1 was also reported by Clozel[31] and Ricou.[32] Although the precise mechanism for the change in the coronary flow distribution induced by ET-1 remains unclear, differences in the transmural distributions or affinities of the ET-1 receptor or the target molecules of ET-1 (for example, ATP-sensitive potassium channels) may explain this interesting phenomenon.[33,34]

Karwatovska-Prokopczuk et al[5] challenged the comparison of endothelin-induced and mechanically induced flow reductions in Langendorff-perfused rabbit hearts, claiming that ET-1 did not exacerbate ischemia. They assessed myocardial ischemia by evaluating oxygen consumption, pH, and purine bodies in the coronary sinus blood. We observed the selective vasoconstriction in the subepicardium using NADH fluorescence; therefore, it might be difficult to assess myocardial ischemia by examining coronary sinus blood. We also tried to measure arterio-venous differences in oxygen and lactate in the coronary circulation but could not identify any ischemia severity between the two treatment groups (data not shown). We also found that some indicators of myocardial ischemia, such as the lactate or creatine phosphate content, were similar in the two treatment groups. Because an increase in NADH and a decrease in the NAD/NADH ratio are the most rapid and sensitive markers for the ischemic redox changes, the method used in the present study can clarify the extent and severity of metabolic changes with a higher sensitivity.

The third unique effect of ET-1 was the appearance of making smaller ischemic foci in the microcirculation, compared with these observations in the stenosis model, as detected by the visualization of many spindle-shaped microischemic foci. Because ET-1 constricts more distal arterioles, as reported by microscopic observation of narrowing small vessels, ET-1 can dominantly regulate coronary microcirculation.[35,36] Under the low flow condition induced by stenosis, subepicardial arteries are maximally dilated, preventing the extension of ischemia. However, the systolic compression of subendocardial arteries limits this compensatory mechanism and induces subendocardial ischemia. A similar size of microischemia was observed in a hemorrhagic shock model.[22]

CONCLUSION

Endothelin-1 produced a larger ischemic area than stenosis in the presence of equivalent reduction in coronary inflow. The mechanism for ET-1–induced ischemia might depend on direct pro-ischemic effects on myocytes and vasoconstriction of the coronary microcirculation, predominantly in the subepicardium in vivo.

ACKNOWLEDGMENTS

The authors thank Mr. Yozo Ohnishi in the Faculty of Medical Equipment of Keio University for his dedicated and excellent technical support and Dr. Motoaki Bessho of the Eisai Tokyo Laboratory for his excellent support in analysis of the metabolites in the myocardial tissues. We also appreciate Mr. Wataru Kawamura of Karl Zeiss Vision Co. Ltd. for his support in analyzing the size of the microischemic foci.

REFERENCES

1. Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;232:411–415.
2. Igarashi Y, Aizawa Y, Tamura M, et al. Vasoconstrictor effect of endothelin on the canine coronary artery: Is a novel endogenous peptide involved in regulating myocardial blood flow and coronary spasm. *Am Heart J*. 1989;118:674–678.
3. Vojacek J, Kolar J, Lisy O, et al. Time course of endothelin-1 plasma level in patients with acute coronary syndromes. *Cardiology*. 1999;91:114–118.
4. Muramatsu K, Tomoike H, Ohara Y, et al. Effects of endothelin-1 on epicardial coronary tone, coronary blood flow, ECG-ST change and regional wall motion in anesthetized dogs. *Heart Vessels*. 1991;6:191–196.
5. Karwatowska-Prokopczuk E, Wennmalm Å. Effects of endothelin on coronary flow, and mechanical performance, oxygen uptake, and formation of purines and on outflow of prostacyclin in the isolated rabbit heart. *Circ Res*. 1990;66:46–54.
6. Fukuda K, Hori S, Kusuhara M, et al. Intracoronary endothelin-1 increases coronary retrograde pressure by constricting arterioles. *Cardiovasc Res*. 1990;24:987–992.

7. Watanabe T, Suzuki N, Shimamoto N, et al. Endothelin in myocardial infarction. *Nature*. 1990;344:114.
8. Grover GJ, Dzwonczyk S, Parham CS. The endothelin-1 receptor antagonist BQ-123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc Res*. 1993;27:1613–1618.
9. Kitayoshi T, Watanabe T, Shimamoto N. Cardiovascular effects of endothelin in dogs: positive inotropic action in vivo. *Eur J Pharmacol*. 1989;166:519–522.
10. Ishikawa T, Yanagisawa M, Kimura S, et al. Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. *Am J Physiol*. 1988;255:H970–H973.
11. Takahashi M, Endoh M. Characterization of positive inotropic effect of endothelin on mammalian ventricular myocardium. *Am J Physiol*. 1991; 261:H611–H619.
12. Maczewski M, Beresewicz A. The role of endothelin, protein kinase C and free radicals in the mechanism of the post-ischemic endothelial dysfunction in guinea-pig hearts. *J Mol Cell Cardiol*. 2000;32:297–310.
13. Bessho M, Tajima T, Hori S, et al. NAD and NADH values in rapidly sampled dog heart tissues by two different extraction methods. *Anal Biochem*. 1989;182:304–308.
14. Hori S, Nakazawa H, Ohnishi Y, et al. A rapid cross-sectioning and freeze-clamping device for the beating canine heart. *J Mol Cell Cardiol*. 1989;21: 203–210.
15. Kurihara H, Yamaoki K, Nagai R, et al. Endothelin: a potent vasoconstrictor associated with coronary vasospasm. *Life Sci*. 1989;44: 1937–1943.
16. Barlow CD, Chance B. Ischemic areas in perfused rat heart: measured by NADH fluorescence photography. *Science*. 1976;193:909–910.
17. Chance B, Williamson JR, Jamieson D, et al. Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and in vivo rat heart. *Biochem Z*. 1965;341:357–377.
18. Karp MT, Rauniop RP, Lovgren TN-E. Simultaneous extraction and combined bioluminescent assay of NAD+ and NADH. *Anal Biochem*. 1983;128:175–180.
19. Shimojo N, Naka K, Nakajima C, et al. Test-strip method for measuring lactate in whole blood. *Clin Chem*. 1989;35:1992–1994.
20. Lowry OH, Rosebrough NJ, Farr AL. Protein measurement with the Folin phenol reagent. *J Biochem*. 1951;193:265–275.
21. Mori H, Haruyama S, Shinozaki Y, et al. New nonradioactive microspheres and more sensitive X-ray fluorescence to measure regional blood flow. *Am J Physiol*. 1992;263:H1946–H1957.
22. Miyazaki K, Hori S, Inoue S, et al. Characterization of energy metabolism and blood flow distribution in myocardial ischemia in hemorrhagic shock. *Am J Physiol*. 1997;273:H600–H607.
23. Xu Y, Sandirasegarane L, Gopalakrishnan V. Protein kinase C inhibitors enhance endothelin-1and attenuate vasopressin and angiotensin II evoked [Ca2+] i elevation in the rat cardiomyocyte. *Br J Pharmacol*. 1993;108: 6–8.
24. Lauer MR, Gunn MD, Clusin WT. Endothelin activates voltage dependent Ca2+ current by a G protein-dependent mechanism in rabbit cardiac myocytes. *J Physiol*. 1992;448:729–747.
25. Khandoudi N, Ho J, Karamazyn M. Role of Na+-H+ exchange in mediating effects of endothelin-1 on normal and ischemic/reperfused hearts. *Circ Res*. 1994;75:369–378.
26. Beyer ME, Slesak G, Nerz S, et al. Effects of endothelin-1 and URL 1620 on myocardial contractility and myocardial energy metabolism. *J Cardiovasc Pharmacol*. 1995;26(Suppl 3):S150–S152.
27. Grover GJ, Sleph PG, Fox M, et al. Role of endothelin-1 and big endothelin-1 in modulating coronary vascular tone, contractile function and severity of ischemia in rat hearts. *J Pharmacol Exp Ther*. 1992;263: 1074–1082.
28. Yuki K, Suzuki T, Katoh S, et al. Endothelin-1 stimulates cardiomyocyte injury during mitochondrial dysfunction in culture. *Eur J Pharmacol*. 2001;431:163–170.
29. Chu L, Takahashi R, Norota I, et al. Signal transduction and Ca2+ signaling in contractile regulation induced by crosstalk between endothelin-1 and norepinephrine in dog ventricular myocardium. *Circ Res*. 2003;92:1024–1032.
30. Holland RP, Arnsdorf MF. Solid angle theory and the electrocardiogram: physiologic and quantitative interpretations. *Prog Cardiovasc Dis*. 1977; 19:431–457.
31. Clozel JP, Clozel M. Effect of endothelin on the coronary vascular bed in open-chest dogs. *Circ Res*. 1989;65:1193–1200.
32. Ricou FJ, Murata K, Oh B-H, et al. Evaluation of inotropic effect of endothelin-1 in vivo. *J Cardiovasc Pharmacol*. 1992;20:671–677.
33. Furukawa T, Kimura S, Furukawa N, et al. Role of cardiac ATP-regulated potassium channels in differential responses of endocardial and epicardial cells to ischemia. *Circ Res*. 1991;68:1693–1702.
34. Miyoshi Y, Nakaya Y, Wakatsuki T, et al. Endothelin blocks ATP-sensitive K+ channels and depolarizes smooth muscle cells of porcine coronary artery. *Circ Res*. 1992;70:612–616.
35. Marcus M, Chilian WM, Kanatsuka H, et al. Understanding the coronary circulation through studies at the microvascular level. *Circulation*. 1990; 82:1–7.
36. Wan W, Kanatsuka H, Akai K, et al. Effects of low doses of endothelin-1 on basal vascular tone and autoregulatory vasodilatation in canine coronary microcirculation in vivo. *Jpn Circ*. 1999;63:617–623.