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Reliability of RT-PCR methods for measuring relative gene expression in mast cells

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Abstract

Three methods to quantify gene transcript levels in mast cells, real-time RT-PCR, competitive RT-PCR and conventional RT-PCR analyses, were compared. Linear regression analysis on five gene transcripts revealed that the mRNA levels measured by real-time RT-PCR analysis were minimally correlated with those by conventional RT-PCR analysis. In addition, differences in the mRNA level between samples measured by conventional RT-PCR analysis were smaller than those by real-time RT-PCR analysis, suggesting that conventional RT-PCR analysis is less sensitive at measuring mRNA levels. Results from competitive RT-PCR analysis correlated closely with those from real-time RT-PCR analysis. When the differences in mRNA level between samples are relatively smaller, however, the correlation tended to be weaker. Real-time RT-PCR analysis has higher reliability, but is expensive. In contrast, competitive RT-PCR analysis is inexpensive, but is weaker at detecting smaller differences in gene transcript level between samples. Therefore, the most appropriate analytical method to measure mRNA levels should be chosen, depending on the experimental conditions.

Keywords: Gene transcripts; PCR; Mast cells

1. Introduction

Examining gene expression in cells is indispensable for physiological and pathophysiological evaluation of cell growth and differentiation. The analysis at the mRNA level is one of the initial steps to evaluate gene expression, followed by analysis at the protein level or that at the transcription level or both. Recent development of PCR-based methods of DNA quantification enabled us to measure the mRNA level easily; especially, quantification by real-time PCR using fluorescent reagent and CCD camera is accurate, sensitive and less laborious (Heid et al., 1996). PCR-based methods for quantification of mRNA level are also useful because a relatively small sample of RNA is needed. It is known to be difficult to recover adequate amount of RNA from mast cells (Gilchrist et al., 1997), which play a crucial role during inflammatory and allergic reactions (Williams and Galli, 2000). Previous studies have used various methods to measure the mRNA levels in mast cells, including real-time RT-PCR (Zhong et al., 2003; Funaba et al., 2003c), competitive RT-PCR (Tachimoto et al., 2000; Funaba et al., 2003a,b) and conventional RT-PCR with

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fewer cycles (Bischoff et al., 1999). However, the interrelationships between the mRNA levels measured by various methods have not been reported. Here, we compared the levels of gene transcripts measured by the real-time RT-PCR method with those measured by the competitive RT-PCR method and by the conventional RT-PCR under the fewer PCR cycle conditions.

2. Materials and methods

Cultures of mouse bone marrow-derived, cultured mast cell progenitors (BMCMCs) were described earlier (Ikeda and Funaba, 2003; Funaba et al., 2003b). BMCMCs were treated with growth/differentiation factors such as stem cell factor (Pepro Tech EC, London, UK), transforming growth factor-β_1 (Becton Dickinson, Bedford, MA) and activin A (provided by Dr. A.F. Parlow through the National Pituitary and Hormone Distribution Program at NIDDK) for 8–48 h. RNA isolation and reverse transcription from BMCMCs were described earlier (Ikeda and Funaba, 2003; Funaba et al., 2003b).

PCR primers for conventional RT-PCR analysis and competitive RT-PCR analysis, which were designed for the PCR primer selection program (http://alces.med.umn.edu/rawprimer.html), are shown in Table 1. PCR for conventional RT-PCR analysis was performed in a total volume of 10 μl containing 10 mM Tris–HCl, pH 7.4, 50 mM KCl, 1.5 mM

Table 1
Sequences of oligonucleotides used for conventional RT-PCR, competitive RT-PCR and real-time RT-PCR

Transcript	Primer	Location (nt)	Sequence (5′–3′)	Product (bp)	Accession
Oligonucleotides for conventional RT-PCR and competitive RT-PCR					
c-kit	5′	1–20	GAGCTCAGAGTCTAGCGCAG	1090	XM194218
	3′	1090–1070	CATATATATCCACTGCTGGTG		
FcεRIβ	5′	223–245	TGGAACAATTGTCTGCTCCGTAC	521	J05019
	3′	743–719	GGTCCCCTGATTCTTATGAATCAAC		
FcεRIγ	5′	100–123	GGAGAGCCGCAGCTCTGCTATATC	471	J05020
	3′	570–549	CTGGCAGCTTTATTGGGGGATA		
mMCP-1	5′	288–312	TCCACACAACAGAGGATAAAAGTCG	315	NM008570
	3′	602–577	TCCCACACAGACCTGGAAGTTATAGT		
mMCP-7	5′	177–198	ACCTACTGGATGCATTTCTGCG	636	NM031187
	3′	812–789	CAAGTAATAGGTGACCCGGGTGTA		
G3PDH	5′	319–338	GTATGTCGTGGAGTCTACTG	729	NM008084
	3′	1047–1028	TACTCCTTGGAGGCCATGTA		
Oligonucleotides for real-time RT-PCR					
c-kit	5′	501–520	CCACGGACCTGACGTTTGTC	63	XM194218
	3′	563–546	CGCGCTTCACGTTTTTGA		
FcεRIβ	5′	351–376	CCGAAAGAAAAAACACATTGTATCTG	74	J05019
	3′	424–406	CCCTGCAGCGATGCTACTG		
FcεRIγ	5′	180–199	CAAGATCCAGGTCCGAAAGG	73	J05020
	3′	252–233	GGTGTTCAGGCCCGTGTAGA		
mMCP-1	5′	376–400	AGCTTGAAGAGAAAGCTGAGTTGAC	84	NM008570
	3′	459–438	TCTTCCCAGGGTCGATAAAGTC		
mMCP-7	5′	361–379	GCTCACTGCGGCACACTGT	66	NM031187
	3′	426–404	CGGAGCTGTACTCTGACCTTGTT		
G3PDH	5′	740–759	CGTGTTCCTACCCCCAATGT	73	NM008084
	3′	812–789	TGTCATCATACTTGGCAGGTTTCT		

$MgCl_2$, 0.2 mM of each dNTP, 1 μM of each primer and 0.35 U of a DNA polymerase mixture of the expand high-fidelity PCR system (Roche, Indianapolis, IN) with cDNA sample. After denaturation at 94 °C for 5 min, the thermal cycling parameters consisted of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 60 s for 25–30 cycles followed by extension at 72 °C for 8 min. The PCR products were separated on 2% agarose gels in 1 × TAE buffer and visualized with ethidium bromide. The bands were detected at the expected size (data not shown). The stained image was recorded by an image analyzer (BioDoc-It™ System, UVP Inc., Upland, CA) and the band intensity was quantified using densitometric analysis by Scion image (Scion Corp., Frederick, MD). The mRNA level was expressed as a ratio to G3PDH, and the mRNA level without growth/differentiation factors was set to 1.

Competitor DNA for competitive RT-PCR analysis is shown in Table 2. It was prepared as a deleted mutant DNA, which was synthesized by overlap extension PCR of native PCR product followed by purification using Suprec-02 column (Takara, Tokyo, Japan). A constant amount of competitor DNA was coamplified with the specific primers with cDNA samples or varying amounts of the target cDNA standard. PCR was performed as described above, except for 35 cycles instead of 25–30 cycles. After electrophoresis of the PCR products and staining gels as described above, the band intensity of the amplified competitor DNA at the expected size and the target DNA at the expected size were measured. The log ratios of the amplified competitor and the target were logarithmically plotted against the initial target in a standard curve and the amount of cDNA in the samples was deduced by linear regression analysis (GraphPad PRISM, GraphPad Software, San Diego, CA). The mRNA level was expressed as described above.

PCR for real-time RT-PCR analysis was performed in a total volume of 25 μl with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of each primer and cDNA. PCR was carried out in an ABI-prism 7700 sequence detector (Applied Biosystems, Foster City, CA), using the following conditions: an initial denaturation step consisted of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. PCR primers, which were designed with the computer program Primer Express (Applied Biosystems, Foster City, CA) using parameters recommended by the manufacturer, are shown in Table 1. In a preliminary study, it was verified that PCR products using these PCR primers migrated at the expected size (data not shown). The levels of cDNA in each sample were determined by using the relative standard curve method. The mRNA level was expressed as described above.

3. Results and discussion

The relationship between relative mRNA levels measured by real-time RT-PCR analysis and those measured by competitive RT-PCR analysis is shown in Fig. 1. When the mRNA levels of a total of five genes, composing cytokine receptor (c-kit), high affinity IgE receptors (FcεRIβ and FcεRIγ) and mast cell proteases (mMCP-1 and mMCP-7), are pooled, both PCR systems were highly correlated ($r = 0.829$). Provided that mRNA levels measured by real-time RT-PCR analysis are accurate and reliable (Heid et al.,

Table 2
Competitor constructs by deleting cDNA segment used for competitive RT-PCR

Transcript	Target		Competitor			Accession
	Product (bp)	Location (nt)	Deleting cDNA segment		Product (bp)	
c-kit	1090	1–1090	520 bp	21–540 nt	570	XM194218
FcεRIβ	521	223–743	199 bp	246–444 nt	322	J05019
FcεRIγ	471	100–571	183 bp	124–306 nt	288	J05020
mMCP-1	315	288–602	113 bp	313–425 nt	202	NM008570
mMCP-7	636	177–812	295 bp	199–493 nt	341	NM031187
G3PDH	729	319–1047	372 bp	339–710 nt	357	NM008084

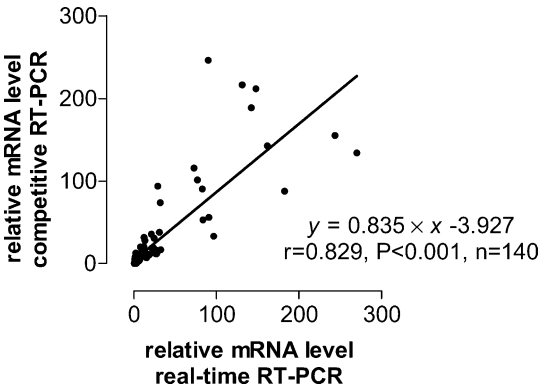

Fig. 1. Overall relationship between relative mRNA levels measured by competitive RT-PCR analysis and real-time RT-PCR analysis in BMCMCs.

1996), these results suggest that competitive RT-PCR analysis is a reliable method to quantify levels of gene transcript in mast cells. An earlier study revealed that DNA levels obtained by the real-time PCR system were comparable with those obtained by the competitive PCR system (Desjardin et al., 1998). These results are consistent with the previous results. However, when the relationship was plotted for individual genes, both measurements were not so well correlated (Fig. 2). In particular, when differences in the mRNA levels between samples were relatively small, no correlation was detected (Fig. 2C). These results suggest that competitive RT-PCR analysis is useful for detecting relatively large differences in mRNA levels between samples.

The relationship between relative mRNA levels measured by real-time RT-PCR analysis and those by conventional RT-PCR analysis is shown in Fig. 3. When the mRNA levels of a total of five genes are pooled, the two systems were correlated. However, the positive relationship between the real-time RT-PCR system and the conventional RT-PCR system was weaker than that between the real-time RT-PCR system and the competitive RT-PCR system (Figs. 1 and 3). In addition, the mRNA levels measured by conventional RT-PCR analysis were clearly lower than

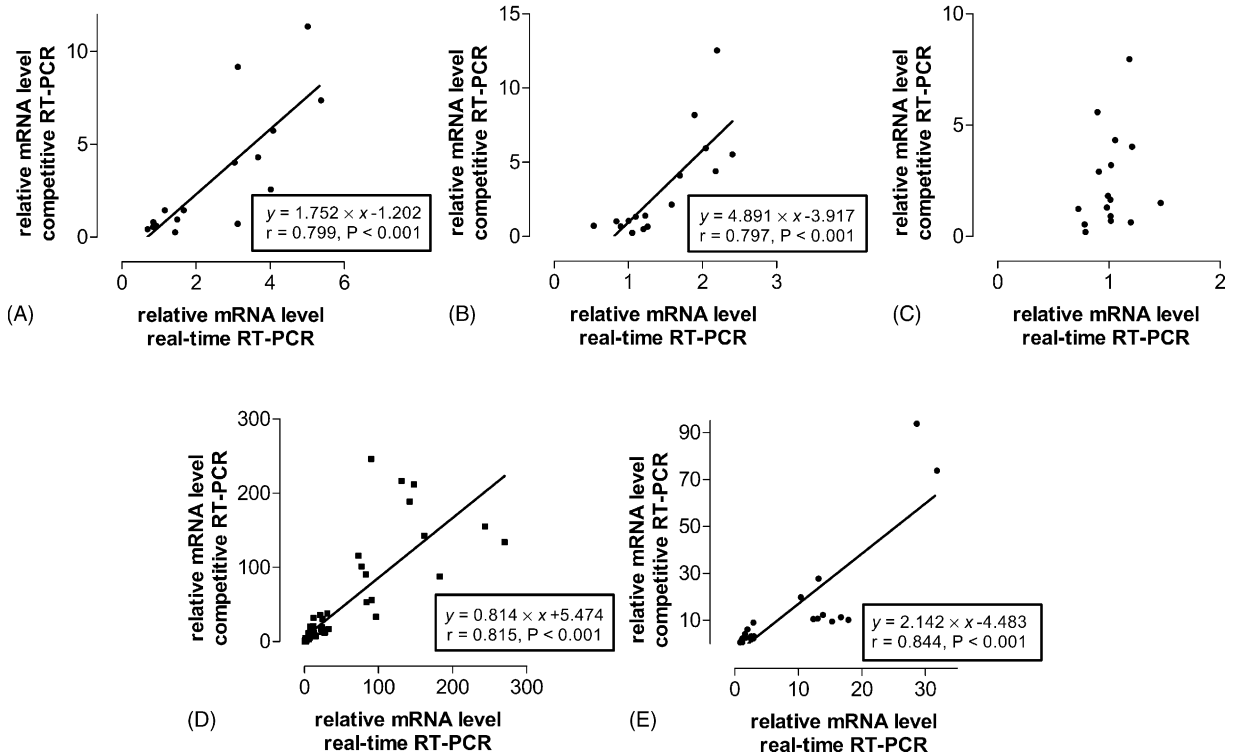

Fig. 2. Relationship between relative mRNA levels measured by competitive RT-PCR analysis and real-time RT-PCR analysis on individual genes, i.e. c-kit (A), FcεRIβ (B), FcεRIγ (C), mMCP-1 (D) and mMCP-7 (E), in BMCMCs.

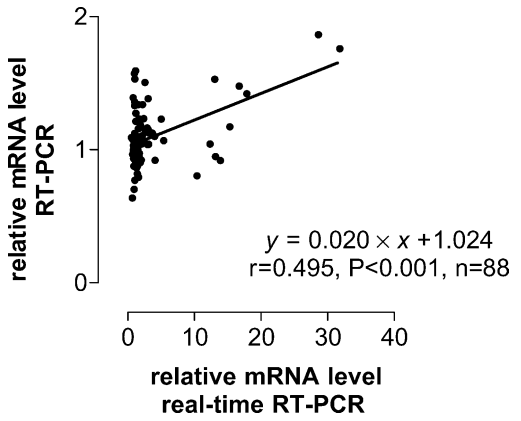

Fig. 3. Overall relationship between relative mRNA levels measured by conventional RT-PCR analysis and real-time RT-PCR analysis in BMCMCs.

those measured by real-time RT-PCR analysis, suggesting that conventional RT-PCR analysis is relatively insensitive for comparing mRNA levels even if PCR is terminated before saturation of the PCR products with fewer PCR cycles.

Real-time RT-PCR analysis is useful in that it is easy, sensitive and accurate in quantifying mRNA levels and it does not require much RNA (Heid et al., 1996). However, special equipment is required to use the system and it is expensive; this is a disadvantage of this system. In contrast, competitive RT-PCR analysis does not require special equipment; it requires an image scanner and analysis software in addition to the PCR machine; the analysis software can be freely downloaded (http://www.scioncorp.com/). However, as compared with real-time RT-PCR analysis, competitive RT-PCR analysis is laborious: preparation of competitor DNA, performing gel electrophoresis of PCR products, gel staining and image analysis are necessary. In addition, the multiple procedures may lead to higher experimental error. In conclusion, both the competitive RT-PCR system and the real-time RT-PCR system can be applied to detect large differences in mRNA levels. In view of its experimental simplicity, use of real-time RT-PCR system is preferable. In contrast, competitive RT-PCR system is recommended for its lower cost. Therefore, depending on the experimental conditions, the more appropriate analytical method for measuring mRNA levels should be chosen.

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